ON LIVER ALDEHYDRASE

by

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Much evidence has been advanced both for and against the identity of aldehyde and xanthine dehydrogenases. Suffice it to say that the work of Booth (1935) and of Dixon and Keilin (1936) has brought conclusive evidence in favour of the view held by Dixon and Thurlow (1924) that in cow's milk one and the same enzyme activates both kinds of substrates, aldehydes and hypoxanthine (or xanthine). In the potato, however, an aldehydrase exists (Bernheim, 1928) which is incapable of activating xanthine.

The corresponding enzyme systems of the liver have been studied much less exhaustively than those of milk, although their physiological importance is greater. Booth (1935) found that the ratio of the activities with salicylaldehyde and hypoxanthine as substrates was the same in enzyme preparations from ox liver and milk, and he concluded from this observation that there was only one enzyme in the liver, identical with that of milk. Reichel and Köhle (1935) came to the conclusion that aldehydes are not directly dehydrogenated in the liver, but that they are dismutated to alcohol and acid, the former of which is then dehydrogenated by the alcohol dehydrogenase.

We became interested in the problem of the liver aldehydrase by some observations made during our study of the reduction of biliverdin by liver dehydrogenase systems (Lemberg and Wyndham, 1936). Aldehydes had been found to be very active hydrogen donators to biliverdin in the presence of slices or enzyme preparations from guinea-pig liver, whereas xanthine was at best a very poor hydrogen donator for the reduction of the bile pigment. One possible explanation of this was the presence of an aldehydrase in the liver, distinct from xanthine oxidase and, in contradistinction to the latter enzyme, able to react with biliverdin as hydrogen acceptor but not with xanthine as hydrogen donator.

For several reasons, however, the experiments with biliverdin did not allow a definite conclusion to be drawn, and the assumption of a separate liver aldehydrase, made in the previous publication, was already based on some experiments to

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be described in this paper. Theoretical considerations, showing that the experiments with biliverdin can be explained perhaps in a different way, will be brought forward later. Additional evidence was also desirable, because the study of the reduction of biliverdin involved special experimental difficulties. The activation of aldehydes towards biliverdin was to a certain extent due to a non-enzymic activation by protein. This complication could be decreased (but not fully prevented) by the use of salicylaldehyde, which is much less activated by the heat-stable system than acetaldehyde. The difference in the biliverdin reduction by salicylaldehyde in presence of unheated and of heated liver enzyme, taken to represent the biliverdin reduction due to the enzymic system alone, was strikingly greater than the very weak reduction of biliverdin by xanthine. The latter was little more than the small reduction obtained by the liver enzyme without addition of substrate.

We have now studied the action of the liver enzyme systems on methylene blue. The rate of reduction of this hydrogen acceptor can be measured by the Thunberg technique much more accurately and conveniently than that of biliverdin. Experiments with methylene blue, moreover, are not subject to the complication of the heat-stable aldehyde activation which was found negligible with methylene blue at 37°.[2]

EXPERIMENTS.

Preparation of Enzymes and Technique.

We have used cow's milk, xanthine oxidase preparations from milk, and enzyme preparations obtained from the livers of man, ox, horse, sheep, guinea-pig, rabbit, echidna, and pigeon.

Fresh cow's milk was procured locally, placed immediately in a refrigerator and investigated within a few hours of delivery. The caseinogen preparation of milk xanthine oxidase was prepared as described by Dixon and Thurlow (1924, a) and stored in an evacuated desiccator. A 10 p.c. solution of the ether-extracted powder in phosphate buffer of pH 7·6 was freshly prepared before the experiments. The "globulin preparation"—as we will call it—of Dixon and Lemberg was prepared as described by Green and Dixon (1934). The yield of this enzyme was much less than when prepared from English milk, but a perfectly water-clear and colourless solution of very high activity was obtained.

The livers of ox, sheep, and horse were procured from the slaughter house or the Zoological Gardens immediately after the animal had been killed; those of rabbits, guinea pig, and pigeon[3] were removed after the animal had been killed and bled. Human liver, in one case, was obtained one hour, in the second case 6 hours after death from uraemia, echidna[3] liver several

[2] Recently Dixon and Keilin (1936) have observed that xanthine oxidase preparations, irreversibly inactivated by incubation with cyanide, still preserved 50 p.c. of their activity in catalysing the reaction between acetaldehyde and oxygen, although they had completely lost their power of catalysing the reactions of hypoxanthine with oxygen or with methylene blue and of acetaldehyde with methylene blue. The remaining ability to activate aldehyde towards oxygen is probably due to the heat-stable protein system which does not measurably activate aldehydes towards methylene blue.

[3] We are indebted to Dr. Abbie (Sydney University) for the specimen of echidna liver, and to Mr. F. A. Moxey for two homing pigeons.

hours after the animal had been killed. The livers were freed from fat and connective tissue, and finely minced in a Latapie mincer. The minced tissue was allowed to stand with about twice its volume of distilled water in the refrigerator for one hour. The extract was centrifuged, and the tissue washed twice with the same amount of water by centrifugation. The combined extracts were half-saturated with ammonium sulphate. The precipitated enzymes were filtered off and dissolved in a small volume of M/10 phosphate buffer of pH 7·6. This solution was dialysed for 24–48 hours in collodion sacs against distilled water. The preparations did not possess any significant action on methylene blue without the addition of substrate.

As substrates we have used salicylaldehyde and xanthine. The latter was prepared from uric acid by the method of Biltz and Beck (1928)—M.P. of the perchlorate was 241° (uncorr.); the Benedict test for uric acid was practically negative. A solution of 2 mg. per c.c. was freshly prepared by dissolving the powder in the minimum quantity of N/20 sodium hydroxide and diluting to volume. Salicylaldehyde was dissolved in phosphate buffer pH 7·6 to give a 0·028 M solution.

The experiments were carried out in Thunberg tubes of the Keilin type with the technique of Green and Dixon (1934). The volume was 7 c.c., including 0·5 c.c. of a methylene blue solution 1: 5,000. The pH was kept at 7·6 with M/10 phosphate buffer.

The Relative Activity of the Enzymes with Xanthine and Salicylaldehyde.

If the assumption were correct that the guinea-pig liver preparations contained an aldehydrase unable to react with xanthine, in addition to xanthine oxidase, it was to be expected that the ratio "*reaction velocity with xanthine*" : "*reaction velocity with salicylaldehyde*" would be found appreciably smaller with liver enzyme preparations than with those of milk containing xanthine oxidase alone.

Booth (1935) has shown that even with one and the same enzyme preparation this ratio is not constant, but depends on the enzyme concentration; this he explained by the fact that the optimal purine concentration varies with the enzyme concentration. We have avoided this mistake by adjusting the enzyme quantities to give approximately the same reduction time with one of the two substrates, unless it was clear from direction and magnitude of the deviation that this could not be explained by differences in enzyme concentration. Xanthine was used in preference to hypoxanthine, since the reaction velocity with the former is less subject to changes of substrate concentration. We have also used different concentrations of substrate (usually 0·1 and 0·3 millimolar xanthine and 1 and 2 millimolar salicylaldehyde) in order to establish the optimal substrate concentration. In general the optimal xanthine concentration was 0·3 millimolar, the optimal salicylaldehyde concentration either 1 or 2 millimolar. The "ratio" given in Table 1 is that of the velocities with xanthine and with salicylaldehyde at optimal concentrations of both substrates.

Table 1 shows that this ratio is not quite constant with one and the same kind of enzyme, but that the results can be clearly classified in two groups with a very great difference in the ratio. With cow's milk and enzymes prepared from it, with ox liver and with human liver, the ratio is above 2, generally between 2 and 3·5.

With the enzymes from the livers of horse, sheep, guinea-pig, rabbit, and echidna, however, much lower ratios were found, usually below 1·0 and often below 0·2.

TABLE 1.

Activity Ratios of Different Enzymes with Salicylaldehyde and Xanthine.

	Enzymes.	Cc. of enzyme used.	Optimal xanthine concentration in millimol.	Reduction time with xanthine in mins.	Optimal salicylaldehyde concentration in millimol.	Reduction time with salicylaldehyde in mins.	Ratio. Decolouration velocity with xanthine/ decolouration velocity with salicylaldehyde
1	Milk, A	2	0·1	4′	2	22·5′	5·6
2	„ B	1	0·3	11′	2	36′	3·3
3	„ C	2	0·3	7·5′	2	16′	2·1
4	„ D	2	2	9′	7	11·25′	1·25
5	„ caseinogen preparation A	2	0·3	6·5′	2	20′	3·1
6	„ caseinogen preparation B	1·5	0·1	4·5′	2	11·5′	2·6
7	„ globulin preparation	1	0·1 = 0·3	2·08′	2	6·83′	3·3
8	Ox liver, A	2	0·3	1·75′	2	3·75′	}
		1	0·3	4·5′	2	9·5′	} 2·1
9	„ „ B	2	0·3	3·25′	2	6·75′	2·1
10	Human liver, A	3	0·3	16′	2	60′	3·75
11	„ „ B	1	0·3	4′	2	15·75′	3·9
12	Pigeon kidney	2	0·3	5·5′	2	95′	17·3
13	Sheep liver, A	1·5	0·1	4′	1	3·25′	0·81
14	„ „ B	2	0·1	9·5′	1 = 2	4·2′	0·44
15	Horse liver, A	1·5	0·3	5·25′	1	1·0′	0·19
16	„ „ B	2	0·3	4·25′	1	2·5′	0·59
17	Guinea-pig liver, A	1	0·3	8′	1	0·75′	0·09
18	„ „ „ B	1	0·3	4·5′	1	3·0′	0·67
19	„ „ „ C	1	0·3	13·5′	1	2·0′	0·15
20	„ „ „ D	1·5	0·3	9·5′	1 = 2	14′	1·47
21	Rabbit liver, A	3	0·1	135′*	1	5·0′	0·03*
22	„ „ B	1	0·3	44′	1	3·3′	0·07
23	„ „ C	1·5	0·3	5′	1	2·5′	0·50
24	Echidna liver	3	0·3	3′	(2)*	1·0′	0·33

* Optimal conc. not determined.

We will call the enzymes with a ratio higher than 2 enzymes of type I, those with a ratio below 1 enzymes of type II. The difference in the ratios cannot be due to variations of the enzyme concentration, since the reduction times with the enzymes of type II were equal to, or shorter than, those with enzymes of type I. A higher concentration of enzymes should (according to Booth) have the effect of increasing the ratio, whereas the opposite effect was actually observed. Expts. 17, 21, and 22 show that some rabbit and guinea-pig livers yield enzyme preparations with a very low ratio, i.e. they are almost unable to react with xanthine whereas aldehydes are rapidly oxidized in their presence. With the livers of different animals of the same species considerable variations were observed, although the animals were kept on the same diet. It should be noted that these variations were

only observed with animals whose liver enzymes are of type II. With guinea-pig in a single case (Expt. 20) a ratio above 1 was observed. Sheep liver yields enzymes of a somewhat higher ratio than those of guinea-pig and rabbit, but still quite different from those of the first class. Horse and echidna liver enzymes also worked faster with salicylaldehyde than with xanthine.

With milk (samples A, B, and C) ratios between 2·1 and 5·6 were obtained. Milk D must be considered as abnormal; the high optima of substrate concentration (both for xanthine and salicylaldehyde) make the presence of an inhibitor

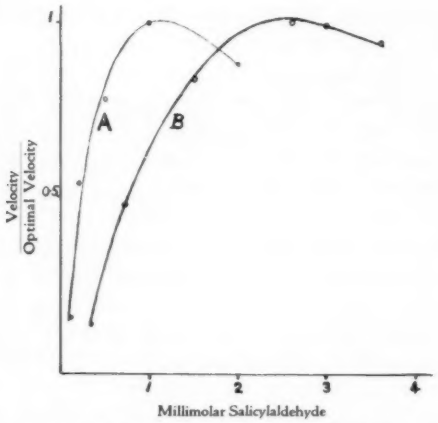

Fig. 1. Substrate concentration curves of guinea-pig liver enzyme (A) and of milk xanthine oxidase (B).

probable. The variations of the enzyme activities in milk concern more the rate of xanthine oxidation than that of aldehyde oxidation and were explained by Dixon as associated with variations of the fat content of the milk. With caseinogen and globulin preparations of xanthine oxidase values of the ratio between 2·6 and 3·3 were observed by us. Since we used xanthine, it is impossible to make an exact comparison of our values with those given by Booth for the ratio of the rate of reaction with hypoxanthine and salicylaldehyde for different enzyme concentrations. In consideration of the fact that hypoxanthine reacts twice as fast as xanthine at low substrate concentrations (Dixon and Thurlow, 1924), a ratio as low as about 1 would be expected from the values given by Booth for the same reaction velocities; but this does not take into account the fact that an excess of xanthine inhibits the reaction less than the same excess of hypoxanthine. The same difference between our values of the ratio and those to be deducted from the values of Booth appears again with ox liver enzyme (2·1 against 1·0—1·3).

Our experiments confirm the result already obtained by Booth that there is no genuine difference in the ratio between milk and ox liver enzyme preparations. Ox liver, however, is the exception rather than the rule, and only in human liver were similarly high ratios found. In confirmation of the observations of Morgan (1926) we found pigeon liver devoid of aldehydrase as well as of xanthine oxidase. In rabbit's kidney, however, we found a small, but distinct, effect of the addition of xanthine and of salicylaldehyde as substrate (reduction time with 0·002 M salicylaldehyde 16 mins., with 0·0003 M xanthine 10·75 mins., control $>30'$). With the pigeon kidney enzyme we obtained a remarkably high value of the ratio (much faster oxidation of xanthine than of aldehyde). Since the enzyme was rather weak—and there is only this isolated observation—we do not attach much evidential value to it.

The differences in the ratios cannot be caused by differences in protein content of the enzyme preparations. The liver enzyme preparations, for example, are much richer in protein than the globulin preparation of xanthine oxidase so that, according to the observations of Booth, an even higher ratio should be expected for liver enzymes than for milk xanthine oxidase.

Table 1 shows that the optimal concentration of salicylaldehyde for oxidation by methylene blue in presence of enzymes of type I was 0·002 M, whereas it was usually 0·001 M with enzymes of type II. Figure 1 shows a comparison of the salicylaldehyde concentration curve of a guinea-pig liver enzyme (ratio 0·25) with that of xanthine oxidase (caseinogen preparation). The latter was taken from the publication of Booth. The reaction velocity, measured in percentage of maximum reaction velocity, is plotted against salicylaldehyde concentration. The substrate concentration giving half maximum velocity is $1{\cdot}6 \times 10^{-4}$ M with the liver enzyme, whereas it is $7{\cdot}8 \times 10^{-4}$ M with xanthine oxidase. (Methylene blue concentration was 4×10^{-5} M, so that the real aldehyde concentration during the decolouration of methylene blue would be somewhat smaller, displacing the curve somewhat more towards the left). The curves do not permit an exact determination of the affinities, since the inhibition by aldehyde probably begins at concentrations below optimal, but this inhibition is not considerable in either case, and hence it can safely be concluded that the liver aldehydrase has a considerably greater affinity for salicylaldehyde than xanthine oxidase.

With acetaldehyde the same results were obtained as with salicylaldehyde (cf. Table 2). The enzymes of guinea-pig and rabbit livers work much more rapidly with acetaldehyde (0·02 M) than with xanthine, whereas milk xanthine oxidase reacts somewhat more rapidly with xanthine. The ratio is somewhat smaller in aged (partially inactivated) than in fresh caseinogen preparation, but with the liver enzymes the ratio is very much smaller than in either, although the activity for xanthine is intermediate. The ratio, "*activity for 0·00054 M salicylaldehyde*":"*activity for 0·02 M acetaldehyde*" is approximately the same for caseinogen preparation (3·1) and guinea-pig liver enzyme (4).

TABLE 2.

Activity Ratios of Different Enzymes with Acetaldehyde and Xanthine.

Concentration of xanthine 0·0004 M, of acetaldehyde 0·02 M.

	Enzyme.	Volume of enzyme used (in c.c.).	Reduction time with xanthine.	Reduction time with acetaldehyde.	Ratio decolouration velocity with xanthine/ decolouration velocity with acetaldehyde.
1	Milk	3	8′	9·5′	1·19
2	„ caseinogen preparation, fresh	3	6′	8′	1·33
3	„ caseinogen preparation, old	3	21·5′	23·5′	1·08
4	Guinea-pig liver	3	11′	0·5′	0·045
5	Rabbit liver	3	16′	1·0′	0·06

Uric Acid Inhibition.

The reaction of xanthine with methylene blue in presence of milk xanthine oxidase is strongly inhibited by uric acid (Dixon and Thurlow, 1924, b). This inhibition decreases with increasing substrate concentration, which shows that substrate and uric acid compete for the active group of the enzyme. The reaction of salicylaldehyde with methylene blue in presence of milk enzyme is also competitively inhibited by uric acid, and this affords strong evidence in favour of the view that both substrates are activated by the same enzyme. Our experiments confirm these results for milk enzyme, and show that similar results are given by the enzymes from ox liver and from human liver, not, however, by the enzymes of other livers.

The experiments were carried out with the Thunberg technique as described above. For the first experiments a solution of uric acid in a minimum amount of dilute sodium hydroxide was used, but it was found difficult to avoid precipitation of uric acid (particularly in presence of the liver enzyme preparations) or shift of the pH (measured electrometrically) to the alkaline side in spite of buffering with phosphate buffer pH 7·6.

We therefore used a solution of uric acid in dilute lithium carbonate (50 mg. uric acid and 30 mg. lithium carbonate in 10 c.c.).

The numbers given in Tables 3–8 are percentages of inhibition (if t is the time of methylene blue decolouration in absence of uric acid, and $t_{ur.}$ the same in presence of uric acid, the percentage inhibition is found as $100\left(1-\frac{t}{t_{ur.}}\right)$. Table 3 shows for milk enzyme and Table 4 for enzyme from ox liver that uric acid competitively inhibits both the oxidation of xanthine and that of salicylaldehyde, and that it inhibits both to approximately the same degree (cf. also Table 6). The same is true for human liver enzyme (Table 5), but the degree of inhibition seems to vary somewhat less with the aldehyde concentration. Those liver enzymes which, like the milk enzyme, react more rapidly with xanthine than with salicylaldehyde (type I) show also the same properties with regard to the uric acid

inhibition which is considerable and decreases with increasing substrate concentration.

TABLE 3.

Uric Acid Inhibition of Xanthine Oxidase from Milk (Caseinogen Preparation).

		Percentage inhibition.					
		0·1	0·3	0·9	1	2	4
		Millimolar xanthine.			Millimolar salicylaldehyde.		
Uric acid	0·009 M	88	79	49	—	—	—
	0·0045 M	—	63	32	67	48	35

TABLE 4.

Uric Acid Inhibition of Xanthine Oxidase from Ox Liver.

		Percentage inhibition.				
		0·3	0·9	1	2	4
		Millimolar xanthine.		Millimolar salicylaldehyde.		
Uric acid	0·009 M	—	—	67	57	35
	0·0045 M	52	16	—	49	28

TABLE 5.

Uric Acid Inhibition of Xanthine Oxidase from Human Liver.

		Percentage inhibition.		
		1	2	4
		Millimolar salicylaldehyde.		
Uric acid	0·0045 M	53	50	44

Those enzymes, however, which react more rapidly with salicylaldehyde (type II) behave quite differently towards uric acid. The oxidation of salicylaldehyde is in general not inhibited by uric acid, but even accelerated. The results, even with one and the same enzyme preparation, vary a good deal more than those with the enzymes of type I. They appear to vary with enzyme concentration. In one case an inhibition was found with guinea-pig liver enzyme, but it was of a non-competitive type (0·009 M uric acid gave 25, 28, and 30 p.c. inhibition, respectively, with 0·001, 0·002, and 0·004 M salicylaldehyde). Sheep liver enzyme stands somewhat intermediate between the two types in so far as uric acid inhibits the aldehyde oxidation, but to a less degree than with the enzymes of type I, and not clearly competitively (0·009 M uric acid gave 33, 50, and 42 p.c. inhibition, respectively, with 0·001, 0·002, and 0·004 M salicylaldehyde; 0·0045 M uric acid gave 33, 35, and 30 p.c. with the same salicylaldehyde concentrations). The acceleration of the aldehyde oxidation by uric acid is not due to uric acid acting as

hydrogen donator; uric acid did not react with methylene blue in presence of any of the liver enzymes.

TABLE 6.

Uric Acid Inhibition of Methylene Blue Reduction by Salicylaldehyde and by Xanthine in Presence of Different Enzymes.

0·009 M uric acid, 0·002 M salicylaldehyde, 0·0003 M xanthine.

Enzyme.	Percentage inhibition of reaction with Salicylaldehyde.	Xanthine.
Milk	67–81	52–77
Caseinogen preparation	67	79
Globulin preparation	65	54
Ox liver	57	49–65
Human liver	55	56
Sheep liver	27–50	35–52
Horse liver	—132*–17	4–35
Guinea-pig liver	—77–28	0–40
Rabbit liver	—75–5	—15–38

* — means percentage acceleration.

On the whole these results seem to confirm the assumption made above that a separate aldehydrase, different from xanthine oxidase, is present in the liver enzymes of type II. A closer study shows, however, that the phenomena are more complex. If the liver enzymes of type II consisted of a mixture of aldehydrase, unable to activate xanthine, with xanthine oxidase, identical with that of milk, then oxidation of xanthine in presence of these enzymes should be inhibited by uric acid to the same degree as in presence of milk enzyme. This, however, is not the case. Like the oxidation of aldehyde, that of xanthine is less inhibited by uric acid in the presence of liver enzymes of type II than in presence of milk enzyme or liver enzymes of type I. In most cases there is still a distinct inhibition of the xanthine oxidation with liver enzymes of type II which, reacting with aldehyde, are not inhibited but accelerated by uric acid (Table 7, Expts. 2, 3, 4, 6, 8, 9); for experiments of Tables 6 and 7 the same enzyme concentration was employed for both xanthine and aldehyde experiments. In other experiments we did not find such a difference, but these can be divided into two classes; in some experiments (1, 7, 10) there was uric acid inhibition of the aldehyde oxidation, as great as that of the xanthine oxidation, but the inhibition of the aldehyde oxidation did not vary with substrate concentration and thus was probably not due to a competition of uric acid with aldehyde for the active group. In other experiments (5, 8, 11) there was neither inhibition of the aldehyde nor of the xanthine oxidation. The degree of uric acid inhibition of the xanthine oxidation with enzymes of type II varies considerably even with one and the same enzyme preparation, like that of the aldehyde oxidation. With horse liver enzyme, we found less inhibition with higher enzyme concentrations (0·0045 M uric acid inhibited the oxidation of 0·0003 M xanthine with 1 cc. enzyme 21 p.c., with 2 cc. enzyme only 7 p.c.).

TABLE 7.

Uric Acid Inhibition of Methylene Blue Reduction with Salicylaldehyde and with Xanthine in Presence of Liver Enzymes.

	Liver enzyme of	Activity ratio xanthine : salicylaldehyde.	Percentage inhibition by 0·009 M uric acid with 0·002 M salicylaldehyde.	0·0003 M xanthine.
1	Sheep	0·81	50*	52
2	Guinea-pig	0·73	—49	36
3	„ „	0·67	—51	24
4	Horse	0·59	—80	35
5	Rabbit	0·54	—17	—15†
6	„	0·50	—75	38
7	Sheep	0·44	27	35
8	Guinea-pig	0·25	—15	2
9	Horse	0·19	—56	17*
10	Guinea-pig	0·14	28*	15
11	Rabbit	0·07	5	2

— designates percentage acceleration.
* Not competitive.
† With 0·1 millimolar xanthine, 19 p.c. inhibition.

When uric acid inhibition of xanthine oxidation was found, it was always competitive with one exception (horse liver enzyme preparation, 9). Examples are given in Table 8.

TABLE 8.

Uric Acid Inhibition of Xanthine Oxidation with Liver Enzymes of Type II.

		Percentage inhibition with xanthine concentrations.		
Enzyme from	Uric acid.	0·0001 M.	0·0003 M.	0·0009 M.
Guinea-pig liver	0·009 M	38	24	—
	0·0045 M	32	15	0
Rabbit liver	0·009 M	—	38	—13
	0·0045 M	19	—15	—

Since these results might be explained by the presence of an inhibitor in the enzyme preparations sufficient to cause maximal or partial inhibition, we have tested the enzyme preparations for the presence of uric acid. If it were already present, a further addition would cause no (or only slight) inhibition according to its concentration. The determination of uric acid with Benedict's reagent, however, showed only minute traces of uric acid in the enzyme solutions, quite insufficient to account for the results obtained.

Apart from the corresponding difference between the types I and II of the enzymes with regard to ratio and uric acid inhibition (cf. Tables 1 and 5) there is no clear correlation between these properties within type II itself, as will be seen from Table 6, where the experiments have been arranged according to decreasing ratio of the reaction velocities. This is, however, not surprising in view of the following considerations. The highest ratio found in those enzymes of type II which were investigated for uric acid inhibition was 0·73. This means that at

the most 24 p.c. of the aldehyde can be activated by xanthine oxidase (if the ratio of this enzyme is 3 and that of the aldehydrase 0). Of this, 65 p.c. can be inhibited by uric acid. The maximal inhibition is thus 16 p.c. This assumes that xanthine oxidase is fully saturated with aldehyde even in the presence of aldehydrase. We have, however, seen that the latter has a much higher affinity for salicylaldehyde than xanthine oxidase, so that the latter will not be saturated with substrate. There is, moreover, a strong, though varying, acceleration of the aldehyde reaction by uric acid in the presence of liver enzymes of type II which will more than counteract a slight inactivation of the xanthine oxidase. It is therefore not surprising that there is no gradual diminution of the uric acid inhibition corresponding to the gradual diminution of the ratio.

DISCUSSION.

Whilst our results confirm the view of the Cambridge school that one and the same enzyme, xanthine oxidase, can activate both aldehydes and purines, and whereas they show that the activation of both kinds of substrate in milk, human liver and ox liver can be satisfactorily explained as due to the action of this enzyme alone, they bear evidence that such an explanation does not suffice for the liver enzyme systems of other species. With these enzymes a relatively much stronger activation of aldehydes is observed than is the case with xanthine oxidase. This result might be interpreted in the following ways:

1. That there are a great number of similar "xanthine oxidases" differing in their relative reaction velocities with xanthine and aldehydes.

2. That there are two different enzymes, one of them activating xanthine only, the other aldehydes only, and all enzyme solutions obtained until now are mixtures of these two enzymes.

3. That there are two different enzymes, one of them xanthine oxidase, activating both substrates with a constant ratio, and found in milk, human liver, and—at least prevailing—in ox liver, the other an aldehydrase accompanying xanthine oxidase and often predominating, in the livers of various animal species.

4. That there is only one enzyme, xanthine oxidase, and that the greater activation of aldehydes with some liver enzymes is due either to a special inhibitor of xanthine oxidation in the livers of type II, or to the presence of a special inhibitor of aldehyde oxidation in milk and livers of type I.

In discussing these possibilities we must also endeavour to explain the facts observed in the study of the uric acid inhibition.

The second assumption can easily be dismissed. Conclusive evidence against it has been accumulated by Dixon and his collaborators, and our results only tend to add to this evidence. The competitive uric acid inhibition of the aldehyde oxidation with methylene blue in presence of milk enzyme and liver enzymes of type I proves that both substrates are activated by the same active centre in these enzymes.

The fourth explanation is very unlikely. As far as the assumption of a special inhibitor of xanthine oxidation in the liver enzymes of type II is concerned, we know of no such inhibitor which inhibits the oxidation of xanthine without also inhibiting the oxidation of aldehydes. In many cases enzymes, reacting much more rapidly with aldehydes, nevertheless have as great an activity with xanthine as that observed with ox liver, which reacts more rapidly with xanthine than with aldehyde. The presumptive inhibitor would have to be undialysable (even on prolonged dialysis we did not observe a change of the ratio), and it would have to prevent the uric acid inhibition of aldehyde oxidation. That such an inhibitor exists is very unlikely. The assumption of a special inhibitor for aldehyde oxidation which would be present in greater concentration in the enzymes of type I than in those of type II does not harmonize with the observed facts either. It does not account for the fact that some liver enzymes, particularly from rabbit livers, are almost inactive with xanthine, nor does it explain the comparative constancy of the ratio in enzymes of type I. This inhibitor also would have to be undialysable and inseparable from xanthine oxidase, since the ratio in the different preparations of the milk enzyme is the same as in milk. Such an alternative can also safely be excluded. As far as we know there are no co-enzymes necessary for the action of xanthine oxidase on either substrate.

The first explanation seems at first sight quite feasible. The existence of similar groups of enzymes with varying relative specificity towards different substrates is known (e.g. glucosidases), although the substrates are generally less different from one another than are xanthine and aldehydes. In our case, however, an attempt to explain the facts on this hypothesis would imply the following assumptions:

In milk and liver enzymes of type I there would be one enzyme (or several very similar enzymes) with a high and little varying xanthine : aldehyde activation ratio, and inhibited by uric acid in practically the same degree and for both kinds of substrate. Then there would be in the livers of type II a great number of different xanthine oxidases, activating both substrates, but with a smaller and widely varying ratio, not inhibited by uric acid for aldehyde, but distinctly, although in a varying degree, for xanthine. Since the ratio differs not only from species to species but also from individual to individual we would be compelled to concede the existence of almost as many enzymes as individuals. This seems highly improbable.

The third possibility remains and accounts satisfactorily for the majority of the facts observed. There is one enzyme, xanthine oxidase, present in milk and in the liver enzymes of type I, either alone or at least considerably prevailing, which is able to activate both substrates. A similar xanthine : aldehyde activation ratio is therefore found with these enzymes, and a degree of uric acid inhibition little varying from enzyme to enzyme, and of the same order for both substrates. In other livers (type II), however, we have besides xanthine oxidase, in a propor-

tion varying even from individual to individual, a second enzyme which activates aldehydes alone. The latter we can conclude from the fact that the ratio is very small in some cases. Particularly in some rabbit livers the aldehydrase prevails so much that the enzyme is practically inactive towards xanthine. The aldehydrase has a greater affinity for salicylaldehyde than the xanthine oxidase, and seems to be less stable, e.g. to cyanide.

Uric acid does not competitively inhibit the action of this aldehydrase, which shows that its active centre does not combine with purines.

It is more difficult to give a fully satisfactory explanation of the facts observed with regard to the uric acid inhibition experiments with liver enzymes of type II. In most cases there was acceleration of the aldehyde oxidation by uric acid, but in a few cases a non-competitive inhibition was observed. Formally one can explain these results as the formation of a compound aldehyde—aldehydrase—uric acid (where uric acid and aldehyde are bound to different places in the enzyme molecule), which reacts more or less (usually more) rapidly with methylene blue than the compound aldehyde—aldehydrase alone. This is little more than a description of facts, but it helps to explain the observation that the reaction with xanthine, as that with aldehydes, is less inhibited by uric acid with enzymes of type II than with those of type I. At first glance this is quite unexpected. Since we assume that xanthine is activated by xanthine oxidase alone in the liver enzyme of type II as in those of type I, it seems difficult to understand why the presence of aldehydrase can have any influence on the uric acid inhibition of the xanthine oxidation. If, however, aldehydrase combines with uric acid, less free uric acid is available to combine with xanthine oxidase, and there is less inhibition of xanthine oxidation by uric acid if the enzyme solution contains aldehydrase.

We assume, therefore that the livers of guinea-pig, rabbit, echidna, and in somewhat smaller amount those of horse and sheep, contain an aldehydrase, not able to react with xanthine; the livers of ox and man, however, do not contain this enzyme. It would be interesting to see whether or not this aldehydrase occurs also in the milk of those animals in the liver of which it occurs.

The following considerations are purely hypothetical, but might be useful as working hypotheses for further study of the specificity problems of this enzyme class. We have found that aldehydes are activated towards biliverdin—much more than towards methylene blue—by amino-acids and proteins (Lemberg and Wyndham, 1936) and, concluding from the results of Lieben and Getreuer (1934), this activation is probably due to the formation of Schiff's bases. We will assume that the proper active centre of an aldehydrase is also the amino-group of amino-acids contained in the protein molecule, the activity of which is considerably increased by other structural factors, and that the aldehydrases are "Hauptvalenz-katalysatoren" like carboxylase (Langenbeck, 1932). The additional factors are due to structural elements which are heat-labile. Together with the amino-group

they form the active principle of the enzyme and, in our case, they also play a rôle in binding methylene blue. The discovery of crystalline proteins with high enzyme activities (pepsin, trypsin, urease) suggests that the enzyme activity in some cases might be caused by a complex spatial arrangement of some quite common constituents of the protein molecule. If this be the case with xanthine oxidase which, according to the way of its preparation, possesses the characteristics of a globulin, then it is possible that the two activating principles of xanthine oxidase and of aldehydrase interpenetrate in the same active group without being identical. We need only assume that the aldehyde-binding amino-group happens to be situated in the structural complex to which the activation of the xanthine is due. In this way one can understand how two kinds of substrates of totally different chemical structure can be activated by one and the same active group, a fact which has hitherto caused some discomfort. The aldehydrase differs perhaps very little from the xanthine oxidase, and only in a few structural points which do not affect its activity towards aldehyde, but destroy that towards xanthine; the separation of two such enzymes from the mixture in which we assume them to be present in the livers of type II may be impossible.

The demonstration of a powerful anaerobic aldehyde dehydrogenase in the liver is of interest with regard to the work of Reichel and Köhle (1935), who came to the conclusion that the oxidation of aldehyde in the liver is caused by the co-action of an enzyme, dismutating aldehyde to acid and alcohol, and an alcohol dehydrogenase. The aldehyde dismutase, prepared by extracting acetone-ether precipitates of liver extracts with water, catalyzed the dismutation of propion-aldehyde, but not its dehydrogenation to propionic acid in presence of either methylene blue or oxygen. Only when rather high concentrations of methylene blue were used, or with oxygen when indophenol oxidase was added, was dehydrogenation of the aldehyde found. These observations are at variance with the facts that xanthine oxidase and liver aldehydrase activate aldehydes towards very low concentrations of methylene blue (4×10^{-5} mol.), and that xanthine oxidase and probably liver aldehydrase also are able to react with molecular oxygen (giving rise to hydrogen peroxide). It is known that xanthine oxidase is destroyed by acetone, and we have found that, with the same treatment by which Reichel obtains his enzymes, 98·5 p.c. of the aldehydrase activity of our preparations of guinea-pig liver enzyme—as measured by the Thunberg technique—was destroyed. It is due to this destruction, and not to the separation of the dismutating enzyme from alcohol dehydrogenase, that Reichel found much less dehydrogenation with his enzyme preparation than with fresh liver. These preparations must be considered to contain damaged enzyme which has to a large extent lost its original properties of combining with methylene blue and molecular oxygen, although it might still be able to activate aldehydes. Even so, the statement of these authors that their enzyme system is unable to react with low concentrations of methylene

blue, is probably incorrect. Their experiments show nothing more than that dehydrogenation did not occur at a measurable rate when the methylene blue : aldehyde equivalent ratio was very low (1 : 7 at the beginning of the experiment). Since aldehyde and methylene blue compete for the enzyme as hydrogen acceptors, this ratio decides how much of the aldehyde is dismutated and how much dehydrogenated. The velocity of dehydrogenation was found almost to equal that of dismutation when the ratio at the beginning of the experiment was one equivalent of methylene blue to two equivalents of aldehyde.[4] This consideration shows that even in Reichel's enzymes the velocities of dehydrogenation and dismutation were of the same order of magnitude. In the fresh liver and in enzyme preparations which have not been damaged by denaturation, the dehydrogenation is certainly much more favoured.

It is probably the liver aldehydrase, and not xanthine oxidase, to which the heat-labile part of the activation of aldehydes against biliverdin by guinea-pig liver enzyme is mainly due, since guinea-pig liver is richer in the former enzyme than in the latter. Xanthine oxidase, moreover, is unable to reduce biliverdin with xanthine. This need not, however, exclude the possibility that xanthine oxidase is also able to activate aldehydes towards biliverdin. Wieland and Mitchell (1932) have observed that, in presence of xanthine oxidase, quinone oxidizes aldehydes more rapidly than xanthine, although methylene blue reacts more rapidly with xanthine. This can be the case if the triple compound xanthine-enzyme-quinone decomposes less readily than aldehyde-enzyme-quinone. In the case of biliverdin the difference between the reaction velocities with the two substrates is much greater than in the case of quinone, but a similar explanation can be given, or it can be assumed that xanthine, but not aldehyde, prevents the access of biliverdin to the active centre. Xanthine inhibits also the access of methylene blue to the active centre more than aldehyde does. Since the lactic acid dehydrogenase system with a much higher redox-potential is able to reduce biliverdin, the xanthine oxidase system is thermo-dynamically capable of doing the same.

Dixon (1926) has shown that xanthine oxidase displays very little specificity with regard to hydrogen acceptors, but it is perhaps significant that, of Clark's reduction-potential indicators, those containing sulphonic acid groups were reduced much less rapidly than those without acid groups. A similar inhibiting effect might be caused by the two propionic acid side-chains of biliverdin. Our experiments indicate that xanthine oxidase can activate aldehydes towards biliverdin to a small degree, since we found heat-labile activation of salicylaldehyde towards biliverdin not only with guinea-pig liver enzyme, but also—although to a minor degree—with milk xanthine oxidase and recently with human liver, which do not contain the aldehydrase.

[4] Since in this experiment a considerable part of the methylene blue was reduced during the period for which the reactions were determined, the average ratio over this total period was in fact still more unfavourable to dehydrogenation than appears from the initial ratio.

SUMMARY.

The activation of aldehydes and of xanthine by liver enzymes is studied with the Thunberg (methylene blue) technique.

For human liver and ox liver the results are in agreement with the assumption of one enzyme, activating both kinds of substrates and identical with the xanthine oxidase of cow's milk.

The livers of other species, however, contain a separate aldehydrase, together with varying amounts of xanthine oxidase.

ACKNOWLEDGMENT.

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THE CHOLINE-ESTERASE ACTIVITY OF BLOOD SERUM FROM TWO CASES OF MYASTHENIA GRAVIS

by

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The success achieved by Dr. Mary Walker (1934) in treating cases of myasthenia gravis with eserine and later with prostigmine, and the confirmation of her work by Blake Pritchard (1935), focussed attention at once upon the possibility of humoral transmission by acetyl-choline in the case of the voluntary nervous impulse.

During 1935 there appeared two papers from the Edinburgh School, which were with that of Ammon and Voss of direct significance in this connection. Ammon and Voss (1935) using a modified Warburg technique and blood rendered non-coagulable, showed that for man, cow, and rabbit, whole blood and its haemolysate are more actively esterolytic than is the serum obtained therefrom; whilst in the case of horse and dog, the reverse was the case. The authors securely established the case for ferment action, as against that which ascribed esterolysis to surface phenomena. They demonstrated that eserine acted similarly upon whole blood, haemolysate, and serum, and thus extended the original observation of Loewy (1930).

What is more significant for the present purposes is their proof that the esterase activity of specimens of human blood drawn from two subjects over a 24-hour period did not alter. They also demonstrated that injection of eserine into a dog caused the esterase activity to drop by 53 p.c. in the case of serum and 80 p.c. in the case of whole blood. This inhibiting effect *in vivo* has been further established by Jones (1935), whilst pilocarpine was shown to be inactive.

Stedman and Stedman (1935) using a Barcroft differential manometer apparatus for the same purpose, found that human blood shows considerable variation in its esterase activity, and in a later paper Stedman (1935) discounts the possibility of excessive choline esterase being a causative factor in myasthenia gravis on the grounds that blood from such patients was shown by his method to contain less esterase. This finding was accepted by Dale (1935) and its validity as a basis of proof that excessive esterolysis might be a causal factor in myasthenia was discussed by Briscoe (1936).

Kruta (1935) using (1) frog's gastrocnemius in Ringer solution, (2) frog's hindquarters perfused with Ringer solution containing $1/10^6$ eserine, and (3) intact animals injected with 0·55–0·1 mg. eserine, could not establish the production of sufficient acetyl choline, on stimulation of the *N. Ischiadicus*, to be responsible for the muscle response, and concluded that this substance could not therefore be the transmitter. Simonart (1935), on the other hand, showed that injection of 0·5–5 mg. of acetylcholine injected into the artery feeding the muscle causes a contraction similar to that produced by Faradic stimulation. This can be repeated for as long as three hours after artificial respiration of the specimen has been started, if 2–3 minutes are allowed for recovery from the effect of the injection. These "refractory periods" become more pronounced as time passes, and both previous injection of acetylcholine and Faradic stimulation at closer intervals than one minute reduce the response to further injection of acetylcholine. It was further shown that the same dose of curare as would block a Faradic stimulation would also block the acetylcholine response.

After 5 mg. acetylcholine direct nervous stimulation is without effect, and a muscle in tetanic contraction is similarly relaxed by acetylcholine injection into the artery. These experimental facts go a long way to support the conclusion that acetylcholine is the probable transmitter of the voluntary nervous impulse, and indicate the point at which Kruta failed to gain such evidence.

A severe case of myasthenia gravis in a young female provided the basis for the present investigation, which quite independently of the work cited, was undertaken in an attempt to establish a relationship between esterase activity and the severity of the symptoms. The results here recorded have been obtained by continuous weekly observation between November, 1935 and October, 1936, with the exception of the month of January. When first seen the patient demonstrated the severest possible symptoms of the disease, and the photographs indicate the effect of an injection of 0·25 mg. of prostigmine in the stage when the respiration was reduced to shallow and ineffective excursions carried on by the accessory muscles —note the sterno mastoids in the first photograph. Anoxia at this stage led to complete loss of consciousness, and one and a half minutes after the injection consciousness had returned with obvious increase of strength in the facial and respiratory musculature. At ten minutes, the patient could smile and hold the eyelids open. These clinical features are given only to establish the true nature of the condition from which the subject was suffering. Full clinical details and results of myographic tests will be reported elsewhere.

On 27th July, 1936, a second case of early myasthenia, as yet not clearly diagnosed as myasthenia gravis, was examined by the same technique. The myasthenia manifested itself by difficulty in pursing the lips to whistle. This weakness increased during the day, and since the possibility existed of myasthenia gravis being present in an early stage, the results are somewhat significant. Blood was drawn aseptically from a normal subject as well as from the patient, and allowed to

Photographs of Case 1.

During attack. Patient unconscious. Respiration by the sternomastoids and serratus muscles.

Three minutes after injection of 1 mg. prostigmine. Eyelids can be momentarily elevated. Respiration by intercostals.

Ten minutes after injection of 1 mg. prostigmine. Eyelids can be held open for some 60 seconds, and subject can smile. Note progressive change in action of circumoral muscles.

clot. The specimens were sent to the laboratory without designation either as to whether from patient or from normal subjects, or as to the clinical condition of the patient. The serum was separated by centrifuging, and tested for its esterase activity by mixing with known concentrations of acetylcholine (Roche). A Straub frog's heart preparation was utilized as test object for most of this work. The surviving frog's rectus according to Gaddum (1933) was also used, as well also as the surviving intestine method of Dale (1914). The Straub preparation was found to be the most satisfactory in that it permitted more rapid handling of the tests, and moreover, in the case of hydrolysis experiments, provided a dynamic picture of the process of esterolysis.

The following are the technical details of the experiments on choline esterase activity as measured by the rate of hydrolysis of known concentration of acetylcholine.

Experimental Details.

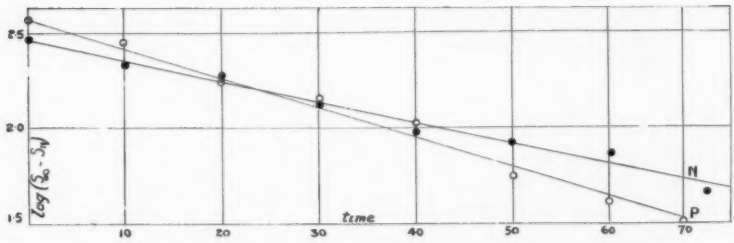
An effort to titrate directly the amount of esterase in the serum, that is to find the volume of serum necessary to destroy a given concentration of acetylcholine in unit time, was made in the first place. This was found to be impracticable because of the relative insensitivity of the frog's heart to acetylcholine during the summer months, and because the sensitivity varied slightly over the working period, making it difficult to fix on a unit of time. To make certain that this latter fact did not give false values for the recovery time during the estimations, the examination of myasthenic serum was immediately followed by that of normal serum. The normal serum used was from different subjects. The concentrations of acetylcholine varied between 1/100,000 and $1/10 \times 10^6$. Most hearts were sufficiently inhibited by concentrations between $1/1 \times 10^6$ and $1/8 \times 10^6$. The hydrolytic activity was estimated by three methods.

1. 0·01 c.c. serum was placed in the empty Straub cannula, and 1 c.c. of acetylcholine of a concentration decided by the sensitivity of the particular heart was added, making the final concentration of serum 1/100. The moment of mixing was noted by marking the drum, and the heart was allowed to recover. The time taken from the mixing of the serum to the recovery of the amplitude of heart beat to a steady level was taken as the recovery time. Fig. 1 shows a typical tracing.

2. 0·02 c.c. of serum and 1 c.c. acetylcholine were mixed, and the time of mixing was marked on the tracing. The serum-acetylcholine mixture was transferred to the cannula after being allowed to react for 1 or 2 minutes. This point was also marked on the tracing, and the heart allowed to recover. In fig. 2, serum and acetylcholine were mixed at a, placed in cannula at b, and the heart beat recovered at c.

3. In this method the hydrolysis was stopped after the action had proceeded for a certain time. This was achieved by a modification of the method described by Matthes (1930). A mixture of 0·1 c.c. of acetylcholine, 1/10,000 dilution; 0·2 c.c. Ringer; 0·1 c.c. serum was taken. The action was allowed to proceed for 1, 2, or 3 minutes, and the esterase action stopped by adding 0·1 c.c. of 20 p.c. trichloracetic acid. The precipitate was centrifuged off, and the supernatant fluid evaporated to dryness. The residue was taken up in alcohol, evaporated to dryness, and finally taken up in 0·5 c.c. Ringer. The acetylcholine content in a 1/10 dilution of this was estimated. Fig. 3.

Fig. 4 shows the effect of prostigmine; 1 mg. of prostigmine was injected into the patient one hour before the sample P_1 was taken. Specimen P_2 was drawn two days later.



Normal serum, $a = -0{\cdot}19$. Patient's serum, $a = -0{\cdot}28$.

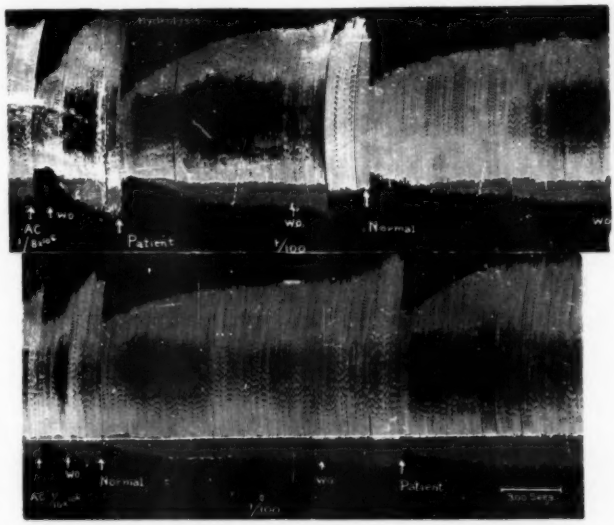
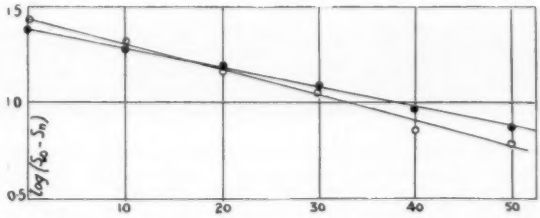


Fig. 1. Hydrolysis of acetylcholine by myasthenic and normal serum. Method No. 1. Blood drawn 8/5/36. Myasthenic condition such that arm could be extended for 10 seconds only. This shows two consecutive readings from same isolated heart, giving same recovery time. Myasthenic is 30 per cent. more rapid than normal.



Normal serum, $a = -0{\cdot}19$. Patient's serum, $a = -0{\cdot}30$.

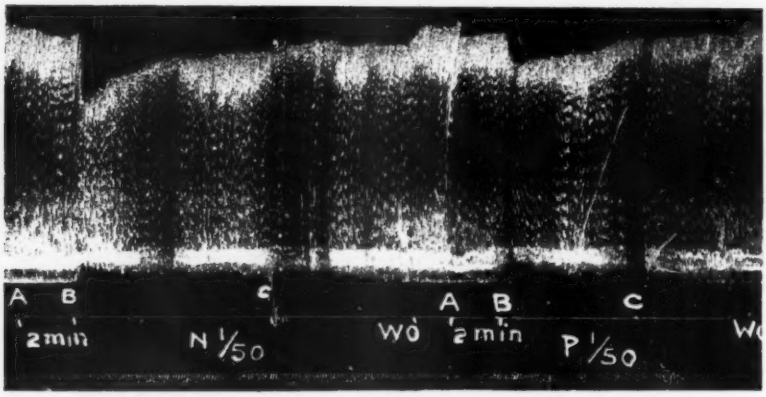
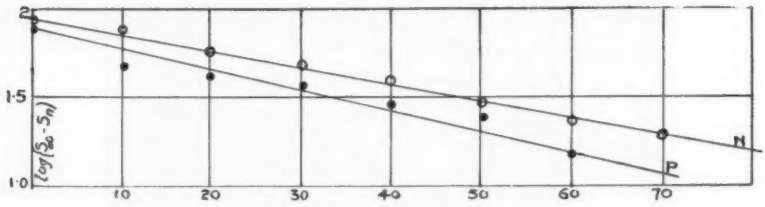


Fig. 2. Hydrolysis of acetylcholine by myasthenic and normal serum. Method No. 2. Blood drawn 18/6/36. No prostigmine given for 20 days. Myasthenic condition fair. Recovery time of heart is 27 per cent. greater than normal.



Normal, $a = -0{\cdot}23$.
Patient, $a = -0{\cdot}17$.

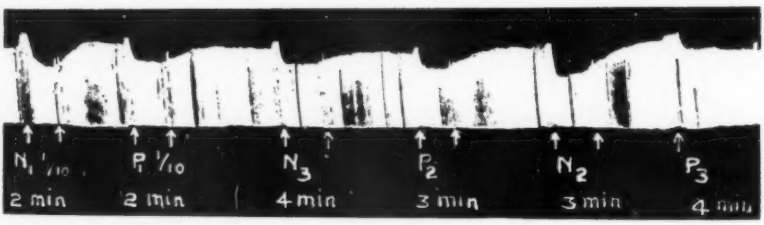


Fig. 3. Hydrolysis of acetylcholine by myasthenic and normal serum. Method No. 3. Blood drawn 22/6/36. Myasthenic condition good. Patient can hold arms extended for 30 seconds. Difference in inhibition of heart between normal and myasthenic estimations is 13 per cent.

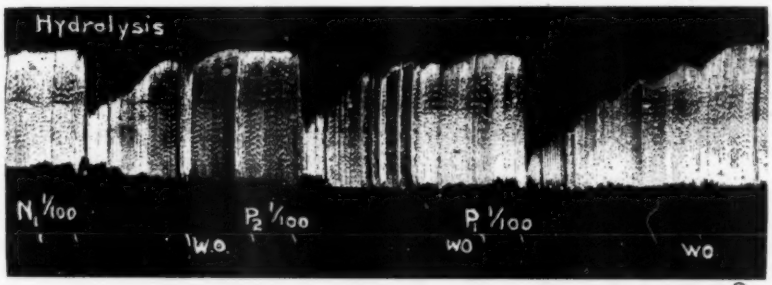
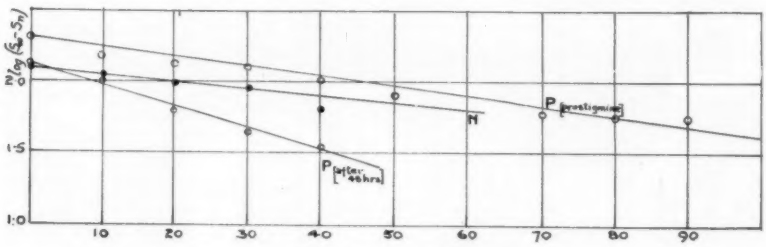


Fig. 4. Hydrolysis of acetylcholine by myasthenic and normal serum, showing the effect of the injection of 1·0 mg. prostigmine. P_1 drawn 17/7/36, 1 hour after the injection of prostigmine; P_2 drawn 19/7/36; and N drawn 18/7/36. Patient very well. Difference in recovery time of heart between normal and myasthenic estimations is 4 per cent.



Normal, $a = -0{\cdot}09$.
Patient + prostigmine, $a = -0{\cdot}23$.
Patient, 24 hours after prostigmine, $a = -0{\cdot}14$.

To differentiate further between myasthenic and normal serum, velocity curves of the recovery of the frog's heart during hydrolysis were plotted.

Working on the assumption that the inhibition of frog's heart corresponds within limits, to the amount of acetylcholine present, and that acetylcholine is destroyed in a monomolecular reaction, so that the curve of recovery is an exponential one, then if S_∞ is the amplitude of the heart beat when the heart is fully recovered, and S_n the amplitude at any point in the curve after a time interval n.

$$\text{Then } S_\infty - S_n = Ke^{an}$$
$$\text{and } \log S_\infty - S_n = k + an$$

Where a = tan of angle of recovery.

This should give a straight line relationship, as is found very approximately to be the case for the first part of the recovery, or if the recovery from maximum inhibition was very rapid. The slope of this curve will represent the rate of esterolyses, and is a negative term because the amount of acetylcholine present decreases with time. If the time of recovery was in any way prolonged, then recovery

took place in two quite definite stages, the reaction velocity during the first stage being less rapid than in the second. The graphs appended show the velocity curves corresponding to the curves illustrating the paper, and were selected from weekly data covering 10 months' work in order to illustrate the results obtained by varying technique, or to document a special clinical experiment. At no time were results obtained which conflicted with the clinical conditions.

It was shown by Abderhalden and Paffrath (1926) that acetylcholine could be resynthesized by the choline esterase which split it into its components, the equilibrium point being at 0·2–0·8 p.c. of acetylcholine. Ammon and Kwiatkowski (1934), using more concentrated sources of esterase (horse serum, Stedman and Easson, 1932) and embryonic extract, have repeated Abderhalden's work, and place the equilibrium point slightly more to the hydrolysis side. Using the technique of Ammon and Kwiatkowski, Shaw (1935) has claimed to have demonstrated that eserine inhibits the hydrolytic phase more than the synthetic phase of this equilibrium.

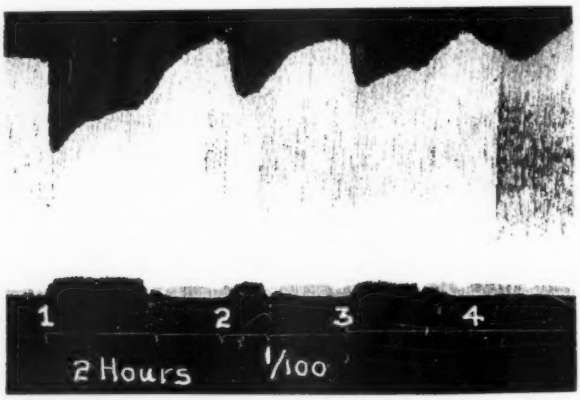


Fig. 5. Synthesis of acetylcholine by normal and myasthenic serum.
1 = Myasthenic serum.
2 = Normal serum.
3 = Normal serum.
4 = Control containing no serum.
Dilution 1/100. Time of incubation, 2 hours.
Blood drawn 18/6/36.

In order to check the results of the hydrolysis experiments, we have utilized the method of Ammon and Kwiatkowski, and carried out both hydrolysis and synthesis measurements of esterase activity simultaneously on the patient's and normal blood serum. The synthesis was carried out by a modification of the method described by Ammon and Kwiatkowski. 0·2 c.c. serum, 0·25 c.c. choline hydrochloride of a concentration of 16·4 p.c., 0·25 c.c. of 16·4 p.c. sodium acetate, and 0·01 c.c. glacial acetic acid were incubated at 38°C., and estimated at hourly intervals. The amount of acetylcholine present was estimated by the degree of inhibition produced in the frog

heart in a 1/100 dilution of the original mixture. A control in which the serum was replaced by 0·2 c.c. from Ringer, was also estimated. Fig. 5 shows a synthesis done when the differences between myasthenic and normal blood were relatively great. This tracing corresponds with the hydrolysis of Fig. 2. Fig. 6 shows the effect of prostigmine and eserine upon the synthesis of acetylcholine. The increasing differences between the amounts of acetylcholine produced by normal and myasthenic serum up to three hours can be seen. The effect of eserine shown in this series is not invariable.

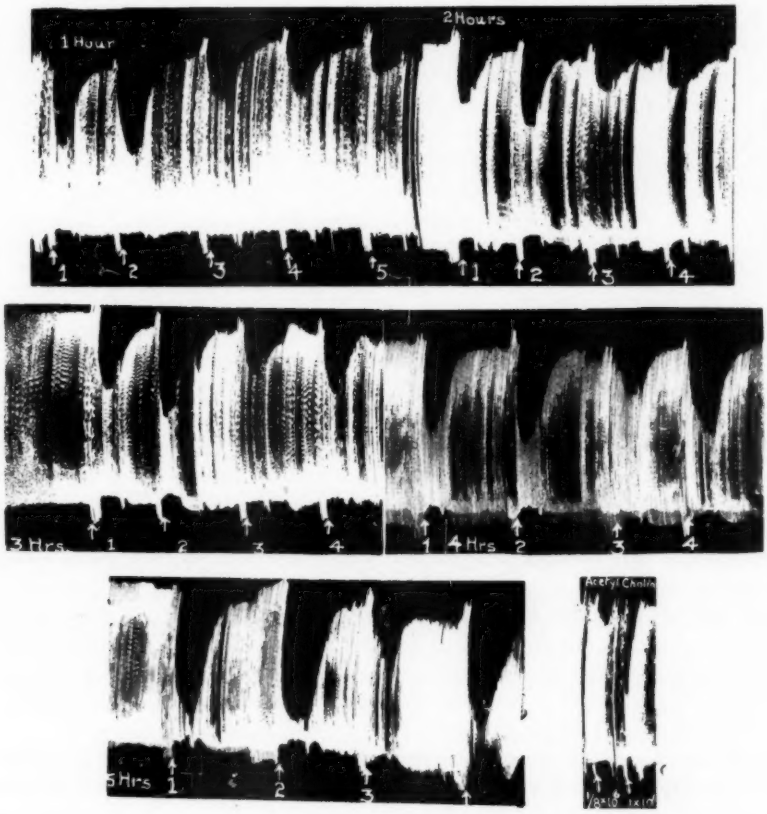
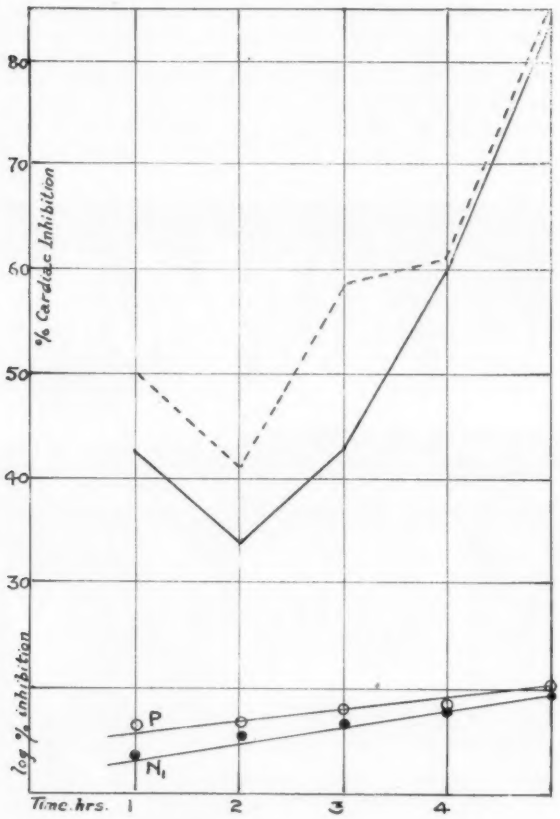


Fig. 6. Syntesis of acetylcholine by normal and myasthenic serum, shewing:

I. The increasing difference in acetylcholine production up to 3 hours.

II. The inhibiting effect of eserine and prostigmine on the synthesis.

1. Normal serum drawn 13/7/36.
2. Myasthenic serum drawn 13/7/36.
3. Myasthenic serum + prostigmine.
4. Myasthenic serum + eserine.
5. Control containing no serum.



Normal, a = — 0·12.
Patient, a = — 0·17.

The results of the hydrolytic experiments have demonstrated consistently greater esterase activity on the part of the patient's blood throughout the eleven months' experimental period, with increased activity during periods of exacerbation and diminution almost to normal during periods of recovery.

The results of the tests of synthetic activity ran uniformly parallel to those of the hydrolytic experiments. The esterase activity of normal sera was found to be constant, and in the case of specimens drawn simultaneously from different subjects the esterase activity was, so far as the method showed, identical. Both prostigmine and eserine sulphate inhibit both phases of the equilibrium, and it

appears from the results of this method of experimentation that the effect of eserine passes off earlier in some instances than does that of prostigmine. This may be due to variations in pH which leave prostigmine unaltered. After injection of 0·26 mg. prostigmine, in the human subject, its effect on esterase activity can be detected by means of the isolated frog's heart within the first two hours at least. This is the period during which, in the case studied, the most marked effect of the drug was noted.

DISCUSSION.

In the cases of myasthenia gravis studied there have been established higher esterase content of serum and variations in the same running parallel with clinical symptoms. Against the possibility of high esterolytic activity of serum (and presumably of tissues) playing a decisive rôle in the causation of the disease, is mainly the fact that the condition is local in its manifestations, if only in the extent of the myasthenia. Inability to purse the lips in order to whistle may be the first symptom noted by the sufferer. How, then, can a generalized alteration in blood esterase be responsible? To answer this question we should require to know the esterase content of the affected tissues, which may be both variable and much higher than the blood esterase indicated. In our case, the latter might well have been an accidental "overflow" index of tissue esterase content, and one not shown in Stedman's series. There may be a local condition associated with a general increase in esterase activity. It is certainly not difficult to picture the breakdown in transmission as being caused by insufficiently rapid accumulation of acetylcholine at the end plate due to increased rate of hydrolysis. Or, if as Dale suggests, *vide* Briscoe (loc. cit.), myasthenia is due to heightened threshold of muscle response to acetylcholine stimulation, the failure to attain this threshold may be due to normal esterase activity which eserine or prostigmine inhibits, thus enabling transmission to take place. Dale's explanation might have been necessary in the cases studied by Stedman. It is too early to conclude from two cases that all cases of myasthenia gravis have the same relation between serum esterase content and symptoms. Even when the esterase activity in our case was only 30 p.c. greater than that of normal, there was extensive myasthenia, so that the quantitative relationship between esterase activity measured in the manner cited, and degree of muscle weakness, is obscure. The conditions in the end plate are, however, unknown, and the end result of a nervous impulse depends on a complex of factors of which rates of formation and destruction of transmitter may provide the main features. This being so, it would be unlikely that a simple quantitative relation would be found to exist between serum esterase activity and the extent of the myasthenia, although parallel relation might well be found to exist as in this case. Since this work had reached the stage when a preliminary report had been published (June, 1936), we have learned (private communication) that Dale, Feldberg, and Vogt (1936) have succeeded in demonstrating that the effect of eserine upon

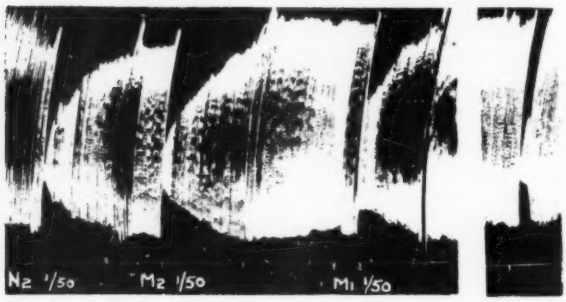
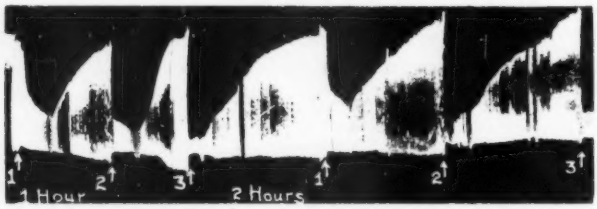
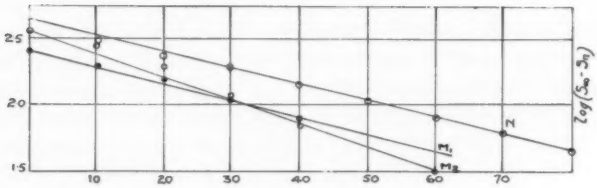


Fig. 7. Second case of myasthenia. Straub frog heart hydrolysis of acetylcholine.
M_1, myasthenic serum drawn 7 a.m.
M_2, myasthenic serum drawn 4.30 p.m.
N_2, normal serum, drawn 4.30 p.m.
Standardization with acetylcholine $1/5 \times 10^6$.

Muscular weakness more pronounced late in day than in the early morning. M_2 is 21·4 per cent. greater than normal. M_1 is 10·8 per cent. greater than normal.



Synthesis. 1 = Normal serum. Straub frog heart.
2 = Myasthenic serum M_1.
3 = Myasthenic serum M_2.
Dilution 1/100. Incubated 1 hour.



Normal, $\alpha = -0{\cdot}23$.
M_1, Patient's serum, 7.00 a.m., no symptoms. $\alpha = -0{\cdot}24$.
M_2, Patient's serum, 4.30 p.m. $\alpha = -0{\cdot}34$. Generalized weakness.

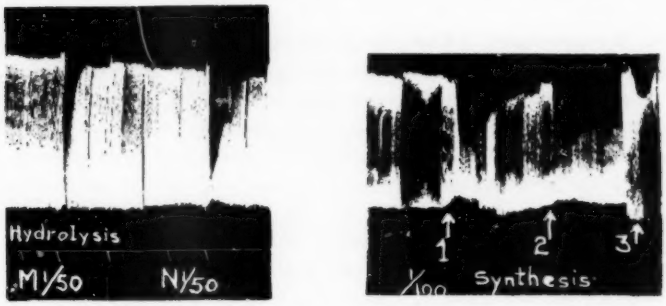
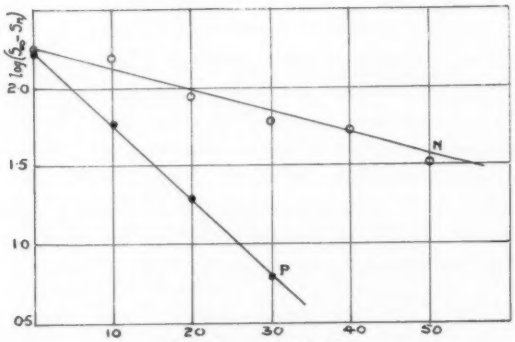


Fig. 8. Second case of myasthenia. Frog heart

Hydrolysis.
M = Myasthenic serum.
N = Normal serum.

Synthesis.
1 = Normal serum. Straub frog heart.
2 = Myasthenic serum.
3 = Control, no serum.

Blood taken at 4 p.m. Difference in recovery time of heart between normal and myasthenic is 22·5 per cent.



Patient, a = — 0·96.
Normal, a = — 0·27.

Synthesis. Eserinized frog rectus abdominis.

1 = Normal Serum.
2 = Myasthenic Serum.
3 = Control.

muscle twitch is to convert it into a stepped and more extensive contraction. Their work and that of Simonart (1935) goes a long way towards establishing the cholinergic transmission of the voluntary nervous impulse, and our own prolonged observations in the present case go to confirm these findings.

After considerable experiment it would appear that both prostigmine and eserine inhibit the hydrolytic and synthetic activity of choline esterase to the same extent, but, in the case of eserine sulphate, the inhibiting effect may pass off much earlier than that of prostigmine, probably owing to unfavourable pH or the presence of organisms. The synthetic activity of normal serum and that of the patient when compared, showed itself in a more rapid production of acetylcholine in the earlier stages, with, however, approximation to identical equilibria after six hours under the conditions of the experiment. This is what would be expected if the esterase activity of normal and patient's blood were in fact quantitatively different, and similar results could be obtained by using variable amounts of serum from the same subject in a synthesis experiment.

Using the technique cited, we have not been able to demonstrate outside the limits of the method any variability in the esterase content of normal blood, either in the same or different individuals, when the same test preparation was used for the assay.

In the second case studied, it will be evident from the tracings and protocols that there is a similar relation between esterase activity and degree of myasthenia (Figs. 7–8). This even extends so far as to indicate, again within the limits of the method, a variation in esterase activity during the course of the day, and one which is, moreover, related to variations in degree of muscle weakness. These results, which were the only original indications that the condition was in fact myasthenia gravis, have since been confirmed clinically, particularly by the action of prostigmine in curing the condition, or preventing its onset.

SUMMARY.

A severe case of myasthenia gravis and an early case of the same disease have been studied continuously for eleven months and four months respectively for choline esterase activity of the blood serum. Simultaneous hydrolytic and synthetic experiments were used in the investigation.

Using the perfused frog's heart, the isolated frog's rectus muscle, and the surviving intestine, evidence has been obtained of increased choline-esterase activity of the serum of the myasthenic patient.

At no time has the patient's choline-esterase activity failed to exceed that of the normal, and it has approached the normal value only when symptoms had almost disappeared. The severity of the symptoms was closely followed by the esterase activity of the serum.

Prostigmine (Roche) and eserine both inhibited the synthetic as well as the hydrolytic action of choline-esterase, but in certain synthesis experiments eserine lost its inhibiting effect. Prostigmine was not subject to this variation of action.

Normal blood serum from the same and from different individuals did not show any variations in choline-esterase content when estimated by means of isolated organs.

ACKNOWLEDGMENTS.

We are indebted to Drs. R. F. West, N. Crosby, and H. R. Oaten for their clinical assistance throughout these investigations.

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METHAEMOGLOBIN, A SPECTROPHOTOMETRIC STUDY

by

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(Submitted for publication 1st October, 1936.)

It has for some time been recognized that methaemoglobin is a compound of haematin with globin, the protein of haemoglobin; this latter substance is a compound of globin with reduced haematin ("haem", "heme", or "hem"), which differs from haematin solely in the lower valency of its iron atom. Methaemoglobin is generally prepared from oxyhaemoglobin by oxidation of the iron with potassium ferricyanide, which is itself reduced to potassium ferrocyanide in the process. In neutral or slightly alkaline solutions an excess of the oxidizing agent is necessary to effect oxidation of all the oxyhaemoglobin present. Like oxyhaemoglobin methaemoglobin is readily converted by dilute acids to acid haematin, a substance in which, as was pointed out by Keilin (1926), the relationship between pigment and protein is probably that of adsorption rather than of chemical combination, the globin functioning as a protective colloid. If such a solution of acid haematin be neutralized a portion of the protein recombines with the pigment to form methaemoglobin. Estimates of the proportion so recovered vary, depending on the conditions of the experiment, the method of determination employed and on whether oxyhaemoglobin or methaemoglobin were used as the source of the acid haematin. Carbon monoxide haemoglobin gives results similar to those obtained with oxyhaemoglobin. Hill and Holden (1926) recovered from oxyhaemoglobin about 40 p.c. of native protein after removal of the haematin with ether and acid and addition of fresh haematin prepared from haemin. They determined the protein in the form of oxyhaemoglobin by comparison of its visible spectrum with that of a known solution of fresh oxyhaemoglobin. Their figures were adversely criticized by Anson and Mirsky (1931) on the grounds of incomplete removal of that denatured protein which was not reconverted into native protein. Anson and Mirsky's method of determination was to estimate the proportion of the protein soluble in a solution containing ammonium sulphate. In one paper (1930, a) they used, for globin, 40 p.c. saturation; in another (1930, b) for methaemoglobin, 50 p.c.; in a third paper (1934) they used

only 30 p.c.; the reasons for choosing these different concentrations are not given. It is the experience of the writer that at 40 p.c. saturation with ammonium sulphate some denatured protein remains in solution protected by the native protein, while a little native protein is carried down with the denatured protein. To what extent these sources of error cancel each other is not clear.

If the pigment and protein be not separated from each other, more methaemoglobin can be recovered if the source of the acid haematin is methaemoglobin than if it is oxyhaemoglobin. The cause of this difference is obscure, but would appear to be associated with the well-known fact that when oxyhaemoglobin is treated with acid some of the oxygen neither oxidizes the iron atom nor appears as gas, but apparently attacks a portion of the protein. The similar results obtained with carbon monoxide haemoglobin suggest a coupled oxidation by gaseous oxygen as the mechanism, since carbon monoxide haem is rapidly oxidized by oxygen to haematin. Wu and Lin (1927) recorded a reconversion to methaemoglobin of all the acid haematin which had been prepared from methaemoglobin. Holden and Freeman (1927), using a spectro-colorimeter, confirmed this. Anson and Mirsky (1934), by the use of sodium salicylate, converted methaemoglobin into parahaematin and obtained a quantitative reversion to methaemoglobin; the protein used is generally described in their paper as "hemoglobin", but it would appear that they really intend to refer to the product of its oxidation. Although they worked at pH 6·8 the parahaematin was not precipitated. They assumed that it was denatured globin parahaematin, and was kept in solution by the presence of the sodium salicylate. This aspect of their hypothesis may need some slight revision. They attributed their success in obtaining a quantitative reversion to native protein to the fact that no precipitate was formed during the denaturation of the methaemoglobin but apparently refrained from an investigation of oxyhaemoglobin on similar lines.

More recently Drabkin and Austin (1936) have studied the spectro-photometry of methaemoglobin in the visible region, using shallow cells and a moderate concentration of the pigment. They record that between pH 6·0 and pH 3·0 their solutions of methaemoglobin were turbid, and that changes in absorption bands, completely reversible on reversing the change of pH, were obtainable only within the range pH 6·05-pH 9·9. An investigation of this rather surprising statement leads one to attribute the turbidity to the presence of hydroferricyanic acid, which is known to have a deleterious action on some proteins. The virtual impossibility of removing quantitatively the complex cyanides after the preparation of methaemoglobin led Hill and Holden (1927) to devise a means of preparing this substance by oxidizing oxyhaemoglobin with ferric alum in slightly acid solution. The methaemoglobin prepared as they describe is free from all inconvenient electrolytes and from denatured protein. It is very stable and, as will be seen, its behaviour differs from that of the methaemoglobin prepared by Drabkin and Austin (1936).

DETAILS FOR GRAPHS.

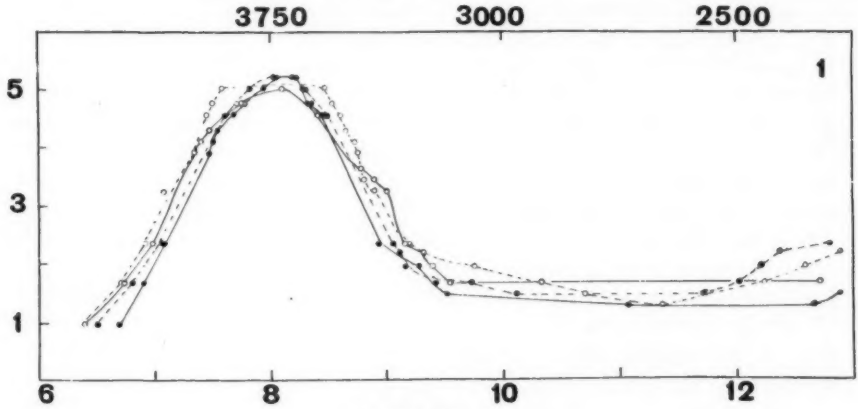
The methods of preparation of some of the solutions are described in the text.

For all Graphs.

Abscissae: $\nu 10^{-14}$ ν is frequency per second.
Ordinates: $\epsilon 10^{-4}$

where $\epsilon = \frac{M}{cd} \log \frac{I_0}{I}$

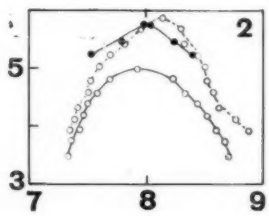
c = No. of grams. of substances in one litre of solution.
d = Depth of cell in cm.
I_0 = Intensity of incident beam.
I = Intensity of emergent beam.
M = Equivalent weight. For haemin, M = 652.
For methaemoglobin, oxyhaemoglobin, acid, and alkaline haematin, M = 16,700.



Graph No. 1.

—— o —— o —— 1 c.c. contains 0·01 mg. haemin and 0·05 mg. gum arabic. pH 2·3.
—— o —— o —— 1 c.c. contains 0·01 mg. haemin and 0·04 mg. gum ghatti. pH 2·3.
—— • —— • —— 1 c.c. contains 0·01 mg. haemin and 0·04 denatured ox serum albumin. pH 2·0.
– – • – – • – – 1 c.c. contains 0·01 mg. haemin and 0·2 mg. denatured ox serum albumin. pH 2·2.

Graph No. 2.



—— o —— o —— 1 c.c. contains 0·134 mg. acid haematin made by adding 2·0 c.c. of 0·1 N. HCl to 0·5 c.c. oxyhaemoglobin solution containing 26·8 mg. per c.c. and diluting to 100 c.c. pH 3·1.

—— • —— • —— 1 c.c. contains 0·1116 mg. acid haematin, made by adding 2·0 c.c. 0·1 N. HCl to 10·0 c.c. containing 1·1116 mg. of methaemoglobin per c.c. and diluting to 100 c.c. pH 3·0.

– – – o – – – o – – – 1 c.c. contains 0·01 mg. haemin and 0·25 mg. irrenaturable ox globin. pH 2·1.

EXPERIMENTAL.

The apparatus used for the spectro-photometry consisted of a Hilger medium spectrograph, a sector photometer and a spark between tungsten steel electrodes. A glass electrode outfit was used to measure the pH of the solutions wherever this quantity is recorded. The haemin used was prepared from ox erythrocytes as described by Hill and Holden (1926). The determination of the proteins was made by coagulation and weighing the dried coagulum.

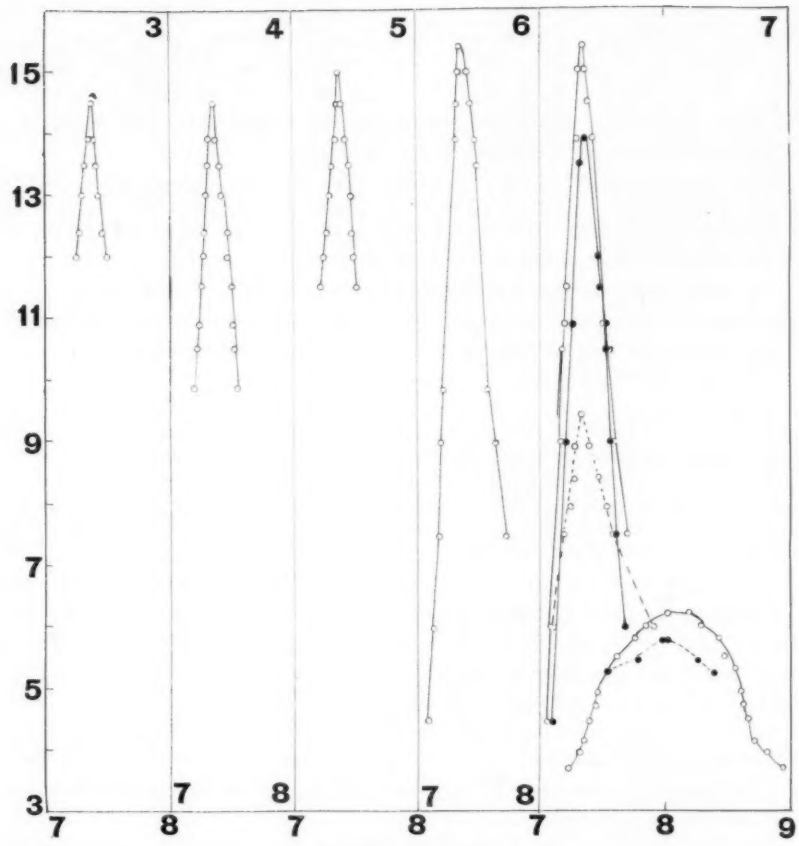
In the experiments no attempt was ever made at a chemical separation between the native and denatured proteins simply because, owing to the tendency of each fraction to include some of the other, the whole advantage of the spectrophotometric method would thus have been thrown away.

A series of spectro-photometric determinations was made on acid haematin prepared from pure haemin and protected by various colloids. In general the results were similar to those obtained by Hicks and Holden (1929), using gelatine as a protective colloid. There was nothing to indicate that any of the various types of colloid used :—gelatine, arabic acid, gum ghatti, irreversibly denatured ox serum albumin, irreversibly denatured ox globin, could so combine with haematin as materially to alter its spectrum. Acid haematin prepared from methaemoglobin and which was afterwards quantitatively "renatured" had the same spectrum as acid haematin protected by a specimen of ox globin from which the haematin had been removed by acid, alcohol and ether and which had been tested for, and found free from, any denatured globin capable of undergoing "renaturation". The maximal intensity of the absorption band decreased after the solution stood for a few hours at 15° C. (Figs. 1-2).

A specimen of ox methaemoglobin was prepared as described by Hill and Holden (1927) and dialysed free from ammonium sulphate. Its gradual conversion to acid haematin with increasing acidity was studied. To avoid possible local effects due to the introduction of acid into protein solutions buffer solutions were used, whenever practicable, to adjust the pH of the solutions before the determinations of the absorption curve were made. It can be seen from the graphs (Figs. 3-7) that under these conditions no appreciable denaturation of the methaemoglobin had occurred up to pH 4·1. Some alkaline methaemoglobin appears to exist as far as about pH 6·3. Conversion into acid haematin is apparent at pH 3·9 and complete by pH 3·0.

Next the renaturation of acid haematin was examined.

Two samples, each 10·0 c.c., of a solution containing 1·116 gm. of methaemoglobin per litre, were placed in 100 c.c. standard flasks. To each was added 2·0 c.c. of 0·1 N. HCl., converting them to samples of acid haematin at pH 3·0. One was made up to 100 c.c., and after 5 minutes its absorption curve determined to show that all the methaemoglobin was denatured. To the other were added successively 78 c.c. water, 8·0 c.c. of sodium citrate solution and enough water to make up to 100 c.c. The sodium citrate solution contained 21·008 gm. of pure crystalline citric acid and 200 c.c. of N. NaOH per litre.



Graphs Nos. 3–7.

No. 3. 1 c.c. contains 0·116 mg. methaemoglobin. pH 7·4.
No. 4. 1 c.c. contains 0·116 mg. methaemoglobin. pH 6·0.
No. 5. 1 c.c. contains 0·116 mg. methaemoglobin. pH 5·3.
No. 6. 1 c.c. contains 0·116 mg. methaemoglobin. pH 4·7.
No. 7.

—— o —— o —— Top curve, 1 c.c. contains 0·1116 mg. methaemoglobin. pH 4·1.
—— • —— • —— 1 c.c. contains 0·1116 mg. mixed methaemoglobin and acid haematin. pH 3·9.
– – – o – – – o – – – 1 c.c. contains 0·1116 mg. mixed methaemoglobin and acid haematin. pH 3·6.
—— o —— o —— Lower curve, 1 c.c. contains 0·1116 mg. mixed methaemoglobin and acid haematin. pH 3.3.
– – – • – – – • – – – 1 c.c. contains 0·1116 mg. haematin. pH 3·0.

The absorption spectra were determined and found equal to those of a control solution containing the same substances at the same reaction, but so mixed that no denaturation had occurred. Thus the renaturation was quantitative. No trace of any precipitate occurred during the experiment. The renaturation in this case was very rapid, being completed within the few minutes occupied in filling the cells and taking the initial photographs (Fig. 8).

In another experiment to 1·0 c.c. containing 0·0558 gm. of ox methaemoglobin were added 2·0 c.c. of 0·1 N. HCl. After five minutes the solution was diluted to 50·0 c.c. with water. Of this solution of acid haematin 10·0 c.c. were taken, 80 c.c. of water added, then 1·6 c.c. of the sodium citrate solution and the solution made up to 100 c.c.

A series of photographs of the absorption band showed that in this case the renaturation was slower than in the former case. After four hours, however, about 95 per cent. of the methaemoglobin had been reconstituted (Figs. 10-13).

Two further experiments were performed as follows:

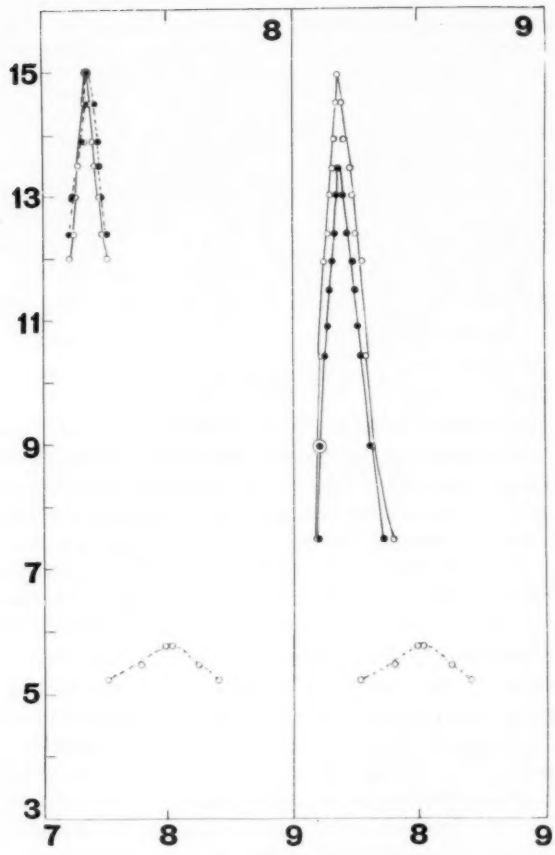
To 0·5 c.c. of this same methaemoglobin solution (55·8 gm. per litre) was added 1·0 c.c. of 0·1 N. HCl. After standing for five minutes 1·0 c.c. of 0·1 N. NaOH was added. The whole of the pigment was immediately precipitated. In one experiment this precipitate was dissolved by the addition of 20 c.c. of water and 0·2 c.c. of 0·16 N. acetic acid and the solution made up to 250 c.c. In the other, 120 c.c. of water and 0·2 c.c. of 0·1 N. NaOH were used to dissolve the precipitate. After two hours 0·3 c.c. of 0·16 N. acetic acid was added and the solution, which remained clear, was made up to 250 c.c.

The absorption was determined and showed that about 90 p.c. of the protein was again in the form of methaemoglobin (Fig. 9). In the case where the precitate was dissolved in acetic acid the renaturation was slower than when alkali was used. After three-quarters of an hour about 60 p.c., after 2½ hours about 80 p.c., and after six hours 90 p.c. was methaemoglobin (Figs. 14-17).

Similar experiments were performed on ox oxyhaemoglobin. Here the proportion of protein that could not be renatured was much larger, 33 p.c. in the case of renaturation in acid solution, and nearly 40 p.c. in the case of renaturation in alkaline solution (Figs. 19, 20).

A determination of the absorption band of ox methaemoglobin prepared from ox oxyhaemoglobin by oxidation with potassium ferricyanide showed the same high band as for the methaemoglobin prepared with ferric alum, even at pH 4·5. The solution used was of course very dilute and the concentration of hydroferricyanic acid in the solution used for the determination correspondingly low. Had any reduction of the methaemoglobin occurred due to the presence of insufficient oxidizing agent in the solution actually used for photography, the effect on the height of the band would have been in the same direction as if a small portion had been denatured by the complex hydrocyanic acids present. That possibility would appear to be excluded (Fig. 19).

Some ox methaemoglobin was denatured by sodium hydroxide at 15° C. At this temperature a higher concentration of alkali than of acid is necessary to denature the protein and even then denaturation is slow.



Graph No. 8.

—— o —— o —— Methaemoglobin denatured by HCl and renatured in very dilute solution. pH 4·7.

— —o— —o— — Acid haematin to show denaturation was complete. pH 3·0.

- - - ● - - - ● - - - Control methaemoglobin. pH 4·7.

Graph No. 9.

—— o —— o —— Control methaemoglobin. pH 4·9.

- - - o - - - o - - - Acid haematin from methaemoglobin. pH 3·0.

—— ● —— ● —— Methaemoglobin renatured in alkaline solution after precipitation. pH for photography 4·9.

A mixture of equal volumes of a solution containing 43·6 gm. of methaemoglobin per litre and of 1·015 N. NaOH was kept at 15°C. After 40 minutes, 10·0 c.c. were withdrawn and diluted to 50·0 c.c. Its absorption curve showed that denaturation was not quite complete. To another 10·0 c.c. sample drawn at the same time were added successively 180 c.c. water, 10·0 c.c. of citrate buffer (0·1 normal, pH 1·92), and enough water to make up to 200 c.c.

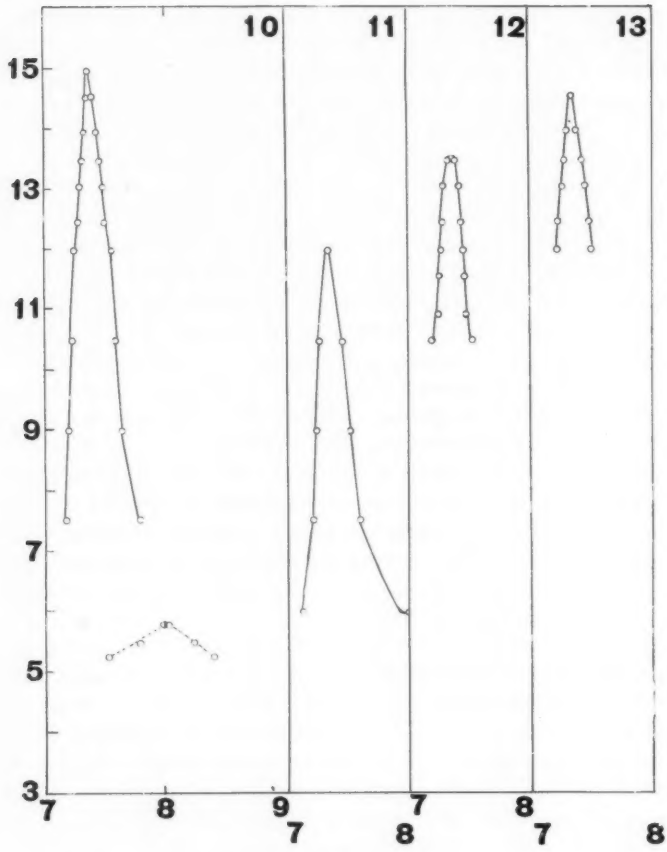
The absorption curve showed that about 30 p.c. of the protein had been renatured. The last traces of alkaline methaemoglobin appeared to be denatured very slowly but after five hours none could be detected either visibly or by the spectro-photometer. At this stage the amount of renaturation which occurred on bringing the solution to pH 4·4 was too small to be shown by the absorption curve. It could, however, be seen with a direct vision spectroscope that a little carbon monoxide haemoglobin was formed by the use of a reducing agent in an atmosphere of carbon monoxide (Fig. 18).

DISCUSSION.

The old conception of a denatured or meta-protein was of one that remains insoluble at its iso-electric point however many times it be dissolved in acid or alkali and reprecipitated by the addition of equivalent amounts of alkali or acid. During the last ten years this idea has been extended, perhaps without many of the workers in this field realizing that it is an extension. Owing to the work of Anson and Mirsky, to which reference has already been made, and of a number of other workers, there are now included in the category of denatured proteins those which are precipitated once at their iso-electric points, but which if suitably dissolved revert, in part at least, to their native originals. Under other conditions they may be dissolved and remain in their own condition and are reprecipitated at their iso-electric points. Indeed the present view would appear to be that this reversible step is the one to which the term denaturation should be restricted, while the word coagulation should cover this step and the one of flocculation, which merely is the act of coming out of solution at the appropriate pH, and which these proteins share with numbers of simpler organic compounds. The change to a form which is permanently that of a meta-protein under the older definition has now no appropriate term and is vaguely referred to under the title of "further changes". The phenomenon of denaturation thus defined is no longer a general property of native proteins, but, contrary to general imagination, is, on the evidence at present available, limited to certain proteins and depends much on the type of chemical used to attack the protein. The lack of terminology makes any adequate discussion exceedingly cumbrous. Perhaps the coining of the word "renature", with its suitable derivatives, to describe the act of converting a denatured protein into a native protein, may add some clarity and save some space. Owing to the fact that in the case of oxyhaemoglobin, on which more of this work has been done than on any other protein, one does not arrive directly at the protein from which one started, but at its oxidation product, the word "reverse" implies more than is

often actually carried out. An irrenaturable protein would then be one which, whether by reason of the properties of the original which was denatured to form it or the agent used for attack, cannot, by the means at present available, be renatured to form any native protein, its original, or an allied native protein. A single, acceptable word for irrenaturable denaturation is needed from the leaders in this field. Ox methaemoglobin (and probably that of other species) can be denatured and renatured quantitatively under suitable conditions. Oxyhaemoglobin of several species can be denatured so that about two-thirds is renaturable while the remaining one-third is an irrenaturable protein. Denatured egg albumin has not been renatured to any significant proportion. Serum albumins of several species have been denatured and the denatured proteins renatured to form albumins indistinguishable from their originals (Spiegel-Adolf (1926), Miller (1933)). In the case of this protein the renaturation appears to be nearly quantitative. If formaldehyde is used to denature a protein no renaturation appears to be possible. There appears to be another class of proteins exemplified by certain vegetable globulins which, in spite of having been irreversibly changed in a direction very like that known as denaturation, retain their ability to crystallize and only betray their departure from their original condition by an increased specific rotation (Freeman, 1934), and by changes in their structure in the solid state as observed with the X-ray spectrograph (Astbury *et alii*, 1935). These last workers suggest that this step be termed degeneration instead of denaturation. Renaturation of a denatured globulin does not seem to have been demonstrated.

From the work in this paper it would appear that the denatured globin parahaematin formed from methaemoglobin is an individual. It can all be renatured to form methaemoglobin again. That formed from oxyhaemoglobin would appear to be a mixture. Hitherto various speculations have been possible concerning the inability of workers to obtain quantitative renaturation of acid haematin prepared from oxyhaemoglobin. The losses have been variously attributed to the effects of reducing agents, alkalies, and to purely mechanical losses. The ultra-violet spectro-photometer avoids all these difficulties and makes it clear that the substance formed by the successive action of acid and equivalent alkali on oxyhaemoglobin consists approximately of two-thirds renaturable globin parahaematin and one-third irrenaturable globin parahaematin. That the action of precipitating the parahaematin is not a serious factor in the proportion of irrenaturable protein produced is shown by the fact that the proportion of renatured methaemoglobin obtained from oxyhaemoglobin is the same whether the parahaematin be precipitated or whether the acid haematin be converted directly to methaemoglobin. Starting from methaemoglobin a small proportion (5 p.c.) is apparently irrenaturably denatured by precipitation, but it is almost within the experimental limits of the instrument used. It is doubtful whether Anson and Mirsky's (1934)



Graphs Nos. 10–13.

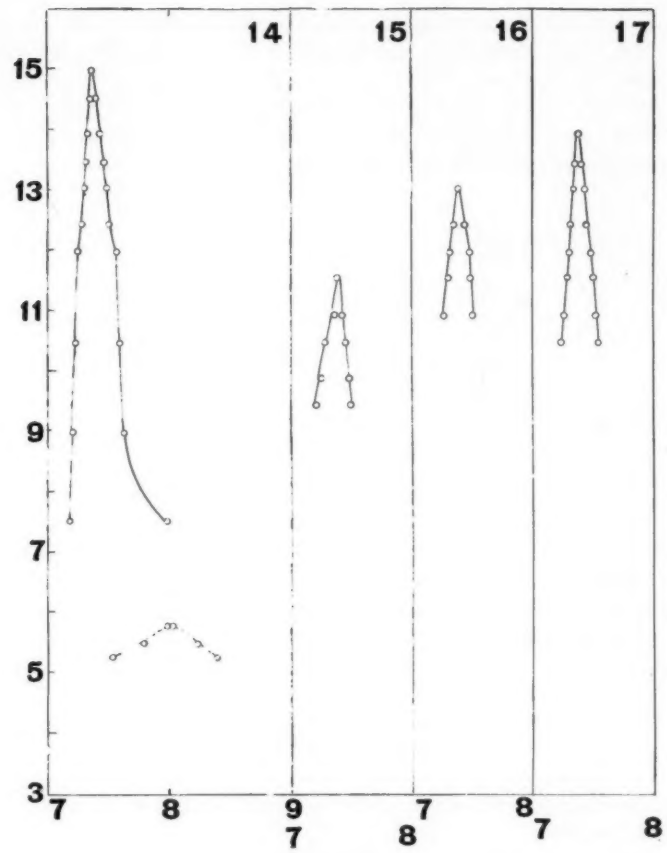
Methaemoglobin denatured in strong solution, diluted, and renatured without precipitation.

No. 10. —— o —— o —— Control methaemoglobin. pH 4·9.
– – – o – – – o – – – Acid haematin from this methaemoglobin. pH 3·0.

No. 11. ¼ hour after bringing solution of acid haematin (Graph 10) to pH 4·9.

No. 12. ¾ hour after No. 11.

No. 13. 3¾ hours after No. 11.



Graphs Nos. 14–17.

Acid renaturation of denatured methaemoglobin after precipitation at the iso-electric point.

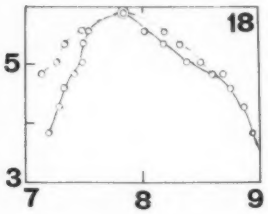
No. 14. —— o —— o —— Control methaemoglobin. pH 4·7.
– – – o – – – o – – – Acid haematin. pH 3·0.

No. 15. ¾ hour after renaturation. pH 4·7.

No. 16. 1¾ hour after No. 15.

No. 17. 5¾ hours after No. 15.

opinion that if precipitation of the denatured protein be avoided quantitative renaturation will follow can remain unmodified. Their success with sodium salicylate as a denaturant for methaemoglobin followed the work of Wu and Lin (1927) and Holden and Freeman (1928) on this pigment, using acid as a denaturant. The quantitative renaturation here would seem to depend rather on the protein selected than on any superiority of sodium salicylate as an agent capable of selectively causing only the first retraceable step.



Graph No. 18.

—— o —— o —— Alkaline haematin.

--- o --- o --- Alkaline haematin 4 hours after adjusted to pH 4·4.

In strongly alkaline solution it would appear that not only is methaemoglobin denatured but that some subsequent change in the configuration of the protein occurs with a velocity relatively high to that of the corresponding reaction in acid solution Hence little of the protein retains its renaturable form for a sufficient length of time to enable much methaemoglobin to be obtained from alkaline haematin, at least by the methods yet available, which are so suitable in the case of acid haematin.

SUMMARY.

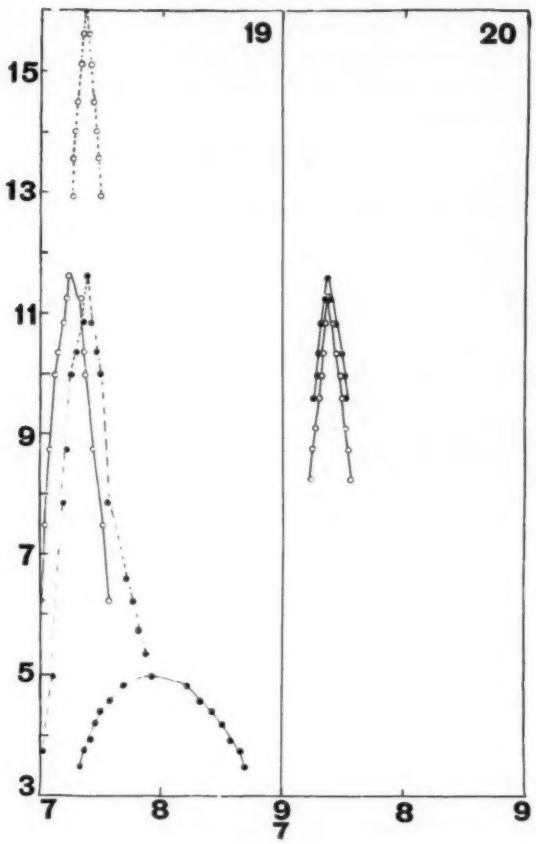
A comparison is made of the spectrum of acid haematin using different types of protective colloids.

The equilibrium between methaemoglobin and acid haematin is examined. Under certain circumstances acid haematin can be quantitatively converted into methaemoglobin.

The preparation from methaemoglobin of a substance insoluble in water which can be reconverted into methaemoglobin is described.

Methaemoglobin denatured by sodium hydroxide yields very little methaemoglobin on bringing the solution to a reaction suitable for its reconstitution.

If ox oxyhaemoglobin be converted into acid haematin not more than about 70 p.c. can be reconstituted to form methaemoglobin under any of the conditions under which acid haematin prepared from ox methaemoglobin undergoes practically quantitative renaturation.



Graph No. 19.

—— o —— o —— Oxyhaemoglobin 0·134 mg. per c.c. pH 6·3.

– – – o – – – o – – – Methaemoglobin by $K_3Fe(CN)_6$ on above oxyhaemoglobin. pH 4·9.

—— • —— • —— Acid haematin from this oxyhaemoglobin. pH 3·1.

– – – • – – – • – – – Renatured methaemoglobin from this acid haematin. pH 5·0. Renaturation was effected without precipitation.

Graph No. 20.

—— o —— o —— Renatured methaemoglobin from acid haematin, fig. 19, after precipitation and solution in sodium hydroxide. pH for photography 4·4.

—— • —— • —— Renatured methaemoglobin from acid haematin, fig. 19, after precipitation and solution in acetic acid. pH 4·3.

ACKNOWLEDGMENT.

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NOTE ON THE DETERMINATION OF PROTEINS IN SOLUTION

by

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(Submitted for publication 15th October, 1936.)

The usual method of the determination of proteins, Kjeldahl's method, though accurate and suitable for a large number of determinations, is laborious for occasional determinations where accuracy is required. Kjeldahl's method cannot be used in the presence of ammonium sulphate, which is often present in protein preparations. To overcome this difficulty the method of heat coagulation in the presence of saturated ammonium sulphate was devised by Hopkins (1900).

Although the method is satisfactory for egg albumin, this is not so with solutions of certain proteins, for example blood pigments and vegetable globulins, the coagulum tending to redissolve as the last traces of electrolyte are being removed.

Observations made in this laboratory have shown that formaldehyde completely denatures proteins and no evidence has been found to show that any portion of this denatured protein tends to redissolve near the iso-electric point of its native original. The effect of heat coagulation in the presence of formaldehyde was studied, therefore, on solutions of the following proteins: egg albumin, edestin, haemoglobin, and ox serum albumin.

It was found that the coagula produced from the various protein solutions by heat in the presence of formaldehyde show no tendency to redissolve on prolonged washing, or heating in distilled water. Coagulation was only effective in the region of the iso-electric point of the protein, and in the absence of other electrolytes a concentration of 0·2 p.c. ammonium sulphate was necessary.

Any absorption of, or combination with, formaldehyde by the protein does not affect significantly the weight of the coagulum obtained. This is shown by the figures in the table. Duplicate determinations were made with the protein coagulated in the presence of a phosphate buffer in the case of oxyhaemoglobin, edestin, and ox serum albumin, and of ammonium sulphate in the case of egg albumin.

Owing to this tendency to redissolve, the coagula produced in the duplicate determinations from edestin, ox serum, albumin, and sheep and rabbit oxyhaemoglobin, were not washed with hot water but thoroughly washed with cold distilled water. 10 c.c. of solution were used for each determination.

10 c.c. of the solution, the protein concentration of which was to be determined, diluted with 30 c.c. water were taken and to the mixture was added 0·5 c.c.

formaldehyde solution (40 p.c. commercial) and 0·2 c.c. saturated ammonium sulphate (if electrolytes were absent); the mixture was heated on a boiling water bath for half-an-hour. The texture of the coagulum thus produced was such that it could be filtered through Gooch crucibles containing asbestos pads covered with a filter paper. The coagulum was filtered while hot, washed with approximately 300 c.c. hot distilled water, finally washed with absolute alcohol and dried to constant weight, the whole process occupying only about two hours, instead of two days by the method using ammonium sulphate only.

Table of Results of Determinations.

EGG ALBUMIN.

Saturated Ammonium Sulphate.	Formaldehyde.	
	Concentration of formaldehyde.	Weight of coagulum.
Weight of coagulum.	0·5 p.c.	0·471 gm.
0·468 gm.		0·474 gm.
0·471 gm.	1·0 p.c.	0·468 gm.
		0·468 gm.
	1·5 p.c.	0·469 gm.

SHEEP OXYHAEMOGLOBIN.

Phosphate Buffer.		Formaldehyde.	
		Concentration of formaldehyde.	Weight of coagulum.
pH 6·0 without formaldehyde.	Weight of coagulum.	0·5 p.c.	0·212 gm.
	0·213 gm.		0·215 gm.
	0·209 gm.	1·0 p.c.	0·208 gm.
			0·209 gm.

RABBIT OXYHAEMOGLOBIN.

Phosphate Buffer.		Formaldehyde.	
		Concentration of formaldehyde.	Weight of coagulum.
pH 6·4 without formaldehyde.	Weight of coagulum.	0·5 p.c.	0·229 gm.
	0·229 gm.		0·230 gm.
	0·229 gm.	1·0 p.c.	0·230 gm.
			0·229 gm.

EDESTIN.

Phosphate Buffer.		Formaldehyde.	
		Concentration of formaldehyde.	Weight of coagulum.
pH 6·8 2·0 p.c. ammonium sulphate	Weight of coagulum.	0·5 p.c.	0·252 gm.
	0·255 gm.		0·257 gm.
	0·252 gm.		

OX SERUM ALBUMIN.

Phosphate Buffer.		Formaldehyde.	
		Concentration of formaldehyde.	Weight of coagulum.
pH 4·8 with ammonium sulphate.	Weight of coagulum.	0·5 p.c.	0·209 gm.
	0·207 gm.		0·206 gm.
	0·208 gm.	1·0 p.c.	0·208 gm.

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A STUDY OF *SPIROCHAETA PENORTHA* (N.SP.) ISOLATED FROM FOOT-ROT IN SHEEP

by

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(Submitted for publication 9th October, 1936.)

In a previous paper (Beveridge, 1935) the finding of a micro-organism, of an unusual type, in lesions of foot-rot in sheep was reported. This organism, which was referred to as the X organism, has now been isolated and studied in pure culture and the name *Spirochaeta penortha* (n. sp.) proposed. It has not been possible to reproduce lesions of foot-rot with pure cultures, or when mixed with culture of *B. necrophorus*, and therefore direct evidence of this organism's causal significance in the disease is still lacking. Nevertheless, its constant association with the disease in our experience, together with certain other indirect evidence, leads us to suspect that it has some aetiological relationship to foot-rot. Failure of cultures to reproduce the disease experimentally may be due in the main to loss of virulence on artificial cultivation, which is a common characteristic of spirochaetes. Further investigation into the pathogenicity of the organism is proceeding. This paper records the results of studies on the organism itself.

ISOLATION.

After numerous failures to isolate the organism by animal inoculation and by the usual bacteriological cultural methods, success was eventually attained by the method used by Noguchi (1912), and modified by Smith (1932), for the isolation of tissue-inhabiting spirochaetes. Briefly, this method is to inoculate in series, with a wire stab, tubes of agar medium containing fresh animal tissue. After two or three weeks' incubation, the spirochaetes grow out through the agar ahead of the bacteria. The process usually has to be repeated several times before a pure culture is obtained.

The organism was also isolated from the same batch of material by making a series of shake cultures in 1 p.c. V-F agar containing 0·1 p.c. of potassium nitrate, 0·1 p.c. of cystein, and 30 p.c. of fresh sheep serum. After six weeks' incubation

the organism was fished in purity from the slightly opaque medium of a tube showing only a few colonies of bacteria.

After the cultural requirements and the resistance to germicides of this strain had been studied, other means of isolation were investigated and finally the following method was evolved. A broth suspension of mixed material containing a large proportion of the organism was exposed to an equal volume of a 1 in 100 solution of trichloracetic acid in distilled water at room temperature for 15 minutes. This was then sown out on 25 p.c. blood agar plates and incubated for one week in an anaerobic atmosphere containing added carbon dioxide, as described later. This method was not uniformly successful, but occasionally some characteristic colonies of the organism were found and these were then subcultured again on blood agar.

MORPHOLOGY.

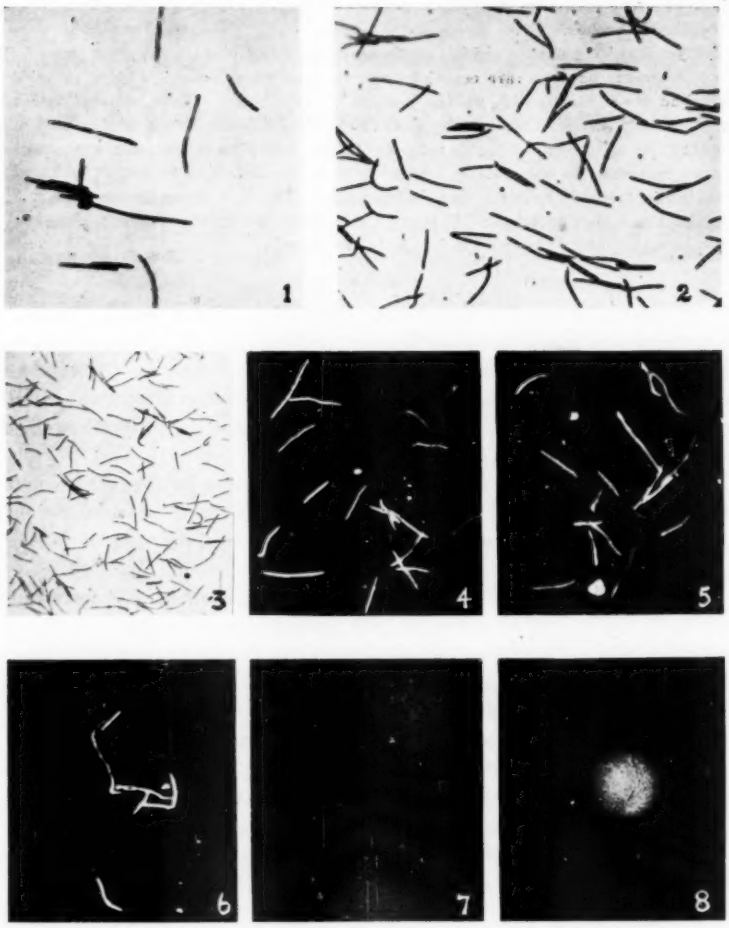
Staining: Aniline gentian violet or strong carbol fuchsin stains the organism in heat-fixed smears quite well within a few seconds, while methylene blue also stains it, but rather more faintly. It is Gram-negative. After fixation with methyl alcohol, Giemsa stains it violet. It usually stains evenly, but sometimes has one or two colourless bands across the filament and rarely may present a beaded appearance when stained with strong carbol fuchsin.

It is a filamentous organism in which neither branching nor spore formation has been observed (Figs. 1–5). It has not been possible to demonstrate either flagella or spirochaetal "terminal filaments" by Zettnow's method. The sides are parallel, though there is sometimes slight tapering at the ends, which are rounded, not pointed. The dimensions of organisms stained by gentian violet or in Congo red preparations, are usually 0·25 to 0·3μ wide by 6 to 10μ long, but may vary from 0·20 to as wide as 0·4μ in culture and as short as from 3μ in young, rapidly growing cultures up to 30μ or even longer in old cultures. In preparations impregnated with silver, the organisms appear twice as wide.

In smears made by spreading material from foot-rot lesions on a slide without moistening it and drying before fixing, filaments of the organism are irregularly curved, and many bend sharply giving various twisted forms, while sometimes a few elements are seen with several more or less regular waves. In smears made from cultures, or from foot-rot material mixed with a drop of fluid on the slide and fixed while wet with osmic acid vapour, the organism is usually only slightly curved, or straight, but occasionally shows very shallow waves.

When observed in the living state under dark ground illumination, the organism appears as a single line of light (owing to its fineness), and is seen to be motile and flexible. The filaments are most often straight, or almost so, but may show curving, which is usually only slight and in one direction, though in old cultures in sloppy agar, long irregularly curved filaments are common. Living specimens do not show the characteristic undulations of spirochaetes, except momentarily sometimes when they encounter an obstacle. However, occasionally on carefully focussing up and down on a non-motile specimen, there can be seen a spiral twist as though the filament were twisted once or twice around its long axis without forming waves.

The organism has a peculiar type of motility. The elements usually progress in a slow, even manner without showing any flexion, unless they come in contact with an obstacle, when they usually bend and may curve around it or reverse their direction. They sometimes perform slightly undulatory movements as they progress in a semi-solid medium, and the advancing end sometimes moves from side to side as though feeling. Occasional individuals actively flex themselves and then straighten out again or curve in the opposite direction. In preparations in which the



Spirochaeta Penortha.

Fig. 1. Smear from growth on blood agar. Osmic acid. Zettnow. (1,500 ×).

Fig. 2. Smear from growth on blood agar. Osmic acid. Gentian violet. (Approx. 1,000 ×).

Fig. 3. Same preparation as in Fig. 2. (650 ×).

Figs. 4 and **5.** Culture in sloppy agar-potato, killed with osmic acid. Dark ground illumination.

Fig. 6. Old culture showing round bodies attached to ends of filaments, one showing a bud-like protuberance.

Figs. 7 and **8.** Surface growth on blood agar. (6 ×).

organisms are very numerous, they tend to adhere together loosely side by side and may move along together in groups.

Highly refractile granules within the filaments are sometimes seen by dark ground illumination, and in smears on very rare occasions may be seen granules which bulge the sides of the filaments and stain deeply with aniline gentian violet. When cultures several weeks old are examined by dark ground illumination, most of the organisms appear granular, and many free granules are also to be seen. Roughly spherical bodies, 0·5–1·5μ in diameter, are sometimes seen in cultures, especially in old cultures. Some of these are attached to one or both ends of the filaments, and a few may have bud-like protuberances (Fig. 6). These bodies stain with aniline gentian violet or carbol fuchsin and have not the staining properties of spores. Noguchi (1912) encountered similar structures with *Spirochaeta phagedenis*.

CULTURAL CHARACTERS.

The organism is a strict anaerobe, and surface growth in an anaerobic atmosphere is improved by the addition of carbon dioxide.

No growth takes place at temperatures about 23° C. Growth at 30-31° C. is definitely slower than at 37° C., while at 33-34° C. growth proceeds well, but not quite as rapidly as at 37° C.

In all media, growth is accompanied by fairly copious gas formation without any very obvious odour, but vigorous cultures have a faint putrefactive smell.

There is no haemolysis of sheep blood added to fluid cultures of the organism nor in blood agar plates with surface growth.

Litmus milk sown heavily is clotted in 2-3 weeks without acid formation.

Growth in Various Media.

No growth takes place ordinarily in unenriched V-F[1] broth or on the surface of V-F agar in air, anaerobically or in an atmosphere with added CO_2, but large inocula sometimes produce growth in V-F broth incubated anaerobically.

In deep V-F agar containing 30 p.c. of sheep serum and fresh guinea-pig kidney, which is a similar type of medium to that used by Noguchi (1912) and Smith (1932) for the routine culturing of spirochaetes, the organism shows growth after one to two weeks' incubation, although in some tubes growth never develops. Growth appears as a fairly dense haze extending from the inoculum out into the agar and up the stab canal. In deep V-F agar containing serum but no fresh tissue, growth sometimes takes place if the medium is heavily seeded.

In V-F serum-broth containing guinea-pig kidney and covered with liquid paraffin, after incubation for a week or more in an anaerobic jar, slight gas production occurs and a small granular deposit forms, sometimes on the side of the tube. Organisms may be difficult to find in smears from the deposit, but can be found in the tissue. Cultures taken from the anaerobic jar show more gas production after they have been left in the air for some time. A similar type of growth occurs outside the anaerobic jar if the medium is boiled immediately before inoculation and the addition of serum and sterile tissue.

[1] V–F media consist of a peptic digest of ox muscle and liver, as described by Turner (1930).

In Lepper and Martin's (1929) cooked heart medium, boiled immediately before use, with or without added serum, growth as shown by gas formation is noticeable after one to five days. The medium later becomes turbid, and after one or two weeks a small, whitish deposit forms. Smears made when gas first appears may not reveal any organisms unless a piece of heart is pressed out on the slide.

Raw Potato Media.

Not only are the foregoing media unsatisfactory owing to delayed growth, but fresh animal-tissue media are difficult to prepare. Raw potato was found to promote a much more rapid and abundant growth than did the guinea-pig or rabbit kidney. Later it was learnt that Weiss and Wilkes-Weiss (1924) had used raw potato media for cultivating *S. pallida*, but they do not mention it as being superior to animal tissue.

The solid medium becomes badly shattered by gas formation and, as cultures in it are not satisfactory for use with animal inoculation, a semi-fluid medium containing 0·2-0·3 p.c. agar was tried. This gave just as good growth without having the disadvantages of the solid medium.

The medium now used for routine cultures is V-F sloppy agar (0·2–0·3 p.c. agar) with raw potato added, referred to as V-F sloppy agar-potato medium, or briefly as V-F S.A.P. To prevent evaporation, liquid paraffin is added if cultures are to be kept in the incubator more than a few days. When this medium is sown liberally growth appears the following day, or, when lightly sown, after three or four days, although lag periods of up to nine days occur after sowing from very old cultures or with very small inocula. Rather bulky, irregular masses of growth form near the potato where the inoculum has been deposited. Abundant gas production forms channels up the medium, and along these the growth spreads. After several days' incubation the growth is carried right to the surface by the gas, and later, if only 0·2 p.c. agar has been used, the medium usually becomes uniformly turbid. Shake cultures give a few spherical colonies which may develop hazy halos.

Serum was added to the S.A.P. medium (S.A.P.S.) in the earlier part of the work, but a comparative test carried out with a strain which had been isolated five months previously showed that growth was equally good when the serum was omitted.

V-F broth, previously boiled, with potato and liquid paraffin added, forms a fairly suitable fluid medium. Some gas production is to be seen after one or two days, and a small to moderate amount of loose, finely floccular deposit forms. The medium becomes turbid if good growth ensues. Unless a fairly heavy inoculum is used no growth may result in this medium.

The use of raw potato in media has greatly facilitated cultural work with the organism: sub-cultures can be obtained overnight instead of at the end of a week or longer as with guinea-pig kidney medium, and the medium can be prepared much more quickly and easily. Raw potato is superior to guinea-pig kidney prob-

ably because it remains alive longer. Boiling for ten minutes destroys the growth-promoting properties of the potato. Filtered potato juice added to media does not promote growth. The principal, and possibly only, action of the raw potato is probably the production of CO_2 and maintenance of anaerobiosis. Cultures in V-F broth in an anaerobic atmosphere with added CO_2 grew as well as did cultures in V-F broth with potato.

The technique for obtaining pieces of sterile, raw potato is as follows: Large potatoes free from injuries are selected, scrubbed, and dried. After some preliminary trimming, a cut surface is seared with a hot spatula. The spatula is heated again and, commencing from the seared surface, a thick slice is cut off thus exposing a sterile surface. The potato is kept under a paper hood to guard against air contamination. With a scalpel sterilized in the flame, pieces of potato are cut out measuring 2 to 3 cm. long by about 0·7 cm. thick, and placed in tubes of medium previously boiled and kept fluid at about 40°C. Although potato has an acid reaction, pieces this size do not appreciably affect the pH of V–F medium, which is adjusted to 7·6.

Growth on Surface of Solid Medium.

No growth has been obtained on serum V-F agar plates incubated aerobically or anaerobically, with or without added CO_2.

On 20 p.c. blood V-F agar plates growth appears after three days' incubation in an anaerobic jar, when they have been sown very heavily from an actively-growing culture. In preparing the plates the blood is added to the melted agar cooled to 42° or 43° C. The addition of large amounts of CO_2 to the atmosphere favours growth. After inoculating the plates, with as little delay as possible they are placed in a McIntosh and Fildes jar. The jar is evacuated and refilled with the inlet held in front of a jet of CO_2. This is repeated, and then about a litre of hydrogen is blown in and the current turned on.

After a week's incubation in an anaerobic atmosphere with added CO_2, confluent growth and some isolated colonies are to be seen on that portion of the plate sown heavily, but not elsewhere. The colonies are convex, and have a glistening surface and, when discrete, an entire edge. Their consistency is butyrous but slightly viscid and, although not very readily emulsifiable, when rubbed up in water they form a homogeneous emulsion which has a marked "watered silk" appearance. They may be wholly opaque or may have a colourless area around an opaque centre. The growth has a characteristic pale green or bluish colour which under a hand lens is seen to be situated in fluorescent granules within the colony (Figs. 7 and 8). Plates sown heavily from previous plate cultures have shown appreciable growth in 24 hours with the typical granular appearance, but white in colour.

VIABILITY AND RESISTANCE.

On three occasions material taken from foot-rot lesions was kept moist in a Petri dish on the laboratory bench for one week, after which time all the spirochaetes were apparently dead, as they had lost their staining properties and motility.

Cultures of the organism in V-F S.A.P. were exposed to air in Petri dishes on the laboratory bench (a) alone, and (b) mixed with an equal volume of *Staph. aureus* culture. The manner in which these cultures died out is shown in Table 1. The organism is destroyed by exposure to air, but is protected to some extent by the presence of *Staph. aureus*.

TABLE 1.

Emulsions of *S. penortha* exposed to air.	Time of Exposure in Days. 1	2	3	4	5	6	7	8	9	11	16
1 ml. alone	—	—									
1 ml. + 1 ml. *S. aureus* culture	+	+	+	—							
3 ml. alone	+	+	—		—						
3 ml. + 3 ml. *S. aureus* culture		+	+		+		—		—		
3 ml. + 3 ml. broth		—		—		—					
3 ml. + 3 ml. *S. aureus* culture				+		+		+		+	—

\+ = *S. penortha* shown to be alive by subculturing.
— = *S. penortha* shown not to be alive by subculturing.

When stored at 4° C. cultures die more rapidly than when kept at 37° C. Three V-F S.A.P.S. cultures kept at 4° C. died in from three to six weeks. Similar cultures lived from 7 to 11 weeks at 37° C., the earliest one to die succumbing in eight weeks, the last one in 12 weeks. Later, on one occasion, two out of a number of stock cultures in V-F S.A.P. kept at 37° C. were found to be dead after four weeks. In guinea-pig kidney serum agar at 37° C. the organism lived as long as 17 weeks in one culture; this solid medium probably maintained the organism longer owing to the air being excluded more efficiently. The viability at room temperature is no greater than at 37° C.

Cultures at the usual pH of 7·6 survived 50° C. for half an hour, 53° C. for 6 minutes, and 55° C. for 2 minutes, but were killed at 53° C. in 9 minutes and 55° C. in 3 minutes.

The organism died in media with pH between 5·0 and 5·6 in 24 hours, but survived at pH 6·0 to 6·3 for 8 days at 37° C., without, however, showing apparent growth. It grew in media containing 1 in 100,000 of neoarsenobillon or a similar concentration of tartar emetic or 1 in 500,000 of gentian violet. But 1 in 100,000 sodium arsenite in media not only prevented growth but killed cultures in 24 hours.

In a series of experiments actively growing V-F S.A.P. cultures were exposed to equal volumes of germicides for 15 minutes at 37° C., and then subcultured into S.A.P. medium while fluid. Under these conditions the organism survived the addition of 1 in 6,000 sodium arsenite, 1 in 1,000 tartar emetic, 1 in 100 trichloracetic acid, 1 in 20,000 gentian violet, 1 in 10 sodium chloride; whereas it was killed by 1 in 4,000 sodium arsenite and 1 in 500 tartar emetic. These are the concentrations of germicides added to the culture, the final dilutions to which the organism was exposed being twice these.

FILTRATION EXPERIMENTS.

Cultures in V-F broth-potato-serum, two and five days old, were filtered through a Pasteur-Chamberland L2 candle at 30 cm. pressure for 30 minutes. The filtrate was sown into V-F S.A.P.S., and the organism grew up five days later. But when culture was filtered through an L2 candle at 20 cm. pressure for 30 minutes no growth occurred in media sown with the filtrate. When fluid cultures were filtered through a Berkefeld N candle at 30 cm. for 30 minutes the filtrate produced no growth.

Material from foot-rot lesions, containing the organism, was suspended in broth and filtered at 30 cm. for 30 minutes on two occasions through L2 candles and once through a Berkefeld N. The filtrates sown into V-F S.A.P.S. gave no growth of the organism, but a small cocco-bacillus grew up in one instance. Broth suspensions of foot-rot material were also filtered through Elford collodion membranes of pore size approximately 0·8μ. In one instance the filtrate was sterile; in the second instance it gave, on culturing, the organism mixed with another small bacterium.

PATHOGENICITY FOR EXPERIMENTAL ANIMALS.

Small doses of mixed material from foot-rot lesions have been inoculated by various routes into sheep, rabbits, guinea-pigs, and mice without the organism becoming established (except, of course, in the feet of sheep).

Guinea-pigs: Four guinea-pigs were inoculated in the thigh, three subcutaneously and one intramuscularly, with from 0·5 to 2·0 ml. of heavy suspension of growth from V–F S.A.P.S. cultures. The inoculation produced a diffuse swelling of the thigh to about twice normal size and, in one animal inoculated subcutaneously, a dark red colour of the skin in addition. The swelling in three of the animals subsided to a nodule the size of a pea one week after inoculation and the animals recovered, though they appeared to have lost weight. The other animal was killed on the third day after being inoculated subcutaneously, and showed an intensely haemorrhagic area in the subcutaneous tissue extending for about an inch around the site of inoculation. Material from this lesion showed only occasional, faintly staining organisms in smear and when inoculated into another guinea-pig it produced only a small, transient swelling. Two guinea-pigs were inoculated intraperitoneally with 2·0 ml. of growth from V–F S.A.P.S. without their health being obviously affected.

Rabbits: One rabbit was inoculated subcutaneously with 0·5 ml. of heavy suspension from a V–F S.A.P.S. culture of the organism. The only lesion produced was a necrotic area 1 × ½ inch, visible through the skin at the site of inoculation. Two rabbits were given series of intravenous inoculations of from 1 to 5 ml. of suspension of growth from fluid cultures without apparent adverse effect. Two rabbits were inoculated intratesticularly with 0·3 and 0·5 ml. of heavy suspension. The testes became swollen and the scrotum of the one receiving the larger dose became oedematous. These testes were removed surgically a week after inoculation, and slightly indurated areas which were found in them were ground up in saline and inoculated into other rabbits' testes. These latter rabbits developed no lesions.

Mice: A mouse was inoculated subcutaneously with 0·35 ml. of heavy suspension of growth from a V–F S.A.P.S. culture. This produced a rather large swelling over which the skin became

very red and later necrotic. The mouse appeared decidedly unwell. The necrotic skin lifted away, leaving an ulcer about 3 mm. across, which healed readily and the mouse recovered. A second mouse was inoculated intraperitoneally with 0·35 ml. of heavy suspension. It appeared unwell for two or three weeks and then recovered.

Sheep: Subcutaneous inoculation of up to 5 ml. of cultures in V–F S.A.P. produced only non-progressive swellings up to one inch in diameter. A preliminary injection of calcium chloride in solution was given to three sheep, 0·2 ml. and 1·0 ml. of 5 p.c. solution, and 2·0 ml. of 2·5 p.c. solution, respectively, and on the following day 0·25, 1·5, and 2·5 ml. of heavy suspension from cultures were inoculated into the same sites. Appreciable swellings resulted from these procedures, the larger doses producing a swelling about ½ inch high and 2 inches in diameter. An attempt was made to passage organisms from these lesions, but the infection died out in the second or third animal in each case.

AGGLUTINATION TESTS.

Suspensions were prepared in carbol saline from growth on blood agar and diluted to an opacity to "Ba III". ("Ba III" standard opacity suspension is made by adding 3 ml. of 1 p.c. barium chloride to 97 ml. of 1 p.c. sulphuric acid.) These suspensions formed a heavy deposit after standing 24 hours at 37° C., but gave little or no deposit at 55° C. for one hour. Suspensions were prepared with two strains which had been isolated in purity, X_1 and X_2, and with one strain X_3 which was contaminated to the extent of about 10-20 p.c. of the organisms in the cultures. (We were not able to free X_3 from this contaminating organism.)

Specific anti-sera against X_1 and X_2 were prepared by inoculating rabbits with a series of injections of from 1 to 5 ml. of heavy growth of organisms in V-F broth-potato-serum.

Each antiserum was put up against each antigen in an agglutination test at 55° C. for one hour, the results being read after the tubes had been standing half an hour at room temperature. These results, which were clear-cut, are shown in Table 2. One normal rabbit serum gave no agglutination with either antigen at a dilution of 1 in 100. As a further control anti-X_1 serum was put up in dilutions of from 1 in 200 to 1 in 32,000 against sheep serum in dilutions 1 in 10, 1 in 100, and 1 in 1,000. No precipitation was observed.

TABLE 2.

Cross Agglutination with Prepared Antisera.

Antiserum.	Antigen.	Final Dilutions of Antisera. 1/1,000	1/2,000	1/4,000	1/8,000	1/16,000	1/32,000
X_1	X_1	. .	+++	+++	++	−	−
	X_2	. .	+++	+++	++	++	+
	X_3	++	++	++	−	−	. . .
X_2	X_1	. . .	+++	+++	++	++	+
	X_2	. . .	+++	+++	++	++	+
	X_3	++	++	++	−	. .	. .

+++ = Complete agglutination.
++ = Agglutination with partial clearing of the fluid.
+ = Agglutination without noticeable clearing of the fluid.
− = No agglutination.

Agglutination tests were carried out with X_2 antigen and sera of six sheep affected with foot-rot and sera of six unaffected sheep. These tests were carried out at 55° C. for one hour, but the tubes were then left at room temperature from 16 to 20 hours before the results were read, as agglutination was often then detectable in higher dilutions. Of the six sera from affected sheep, two agglutinated at a final dilution of 1 in 80, five at 1 in 40, and all at 1 in 20. Of the six sera from normal sheep, one agglutinated at 1 in 80, three at 1 in 20, and all at 1 in 10. Tests put up with X_3 antigen against the sera of the six affected sheep gave results almost identical with those obtained with X_2 antigen.

The results of these tests do not show constantly higher titres in the case of sheep affected with foot-rot. This is perhaps not surprising in view of the strictly localized and relatively superficial nature of foot-rot lesions.

CLASSIFICATION.

Spirochaetes are usually defined as filamentous organisms characterized by (i) spiral form, (ii) flexibility, and (iii) power of movement without flagellae, i.e. by rotation and flexion. Flexibility and spiral form are shown by some bacteria other than spirochaetes (Dobell, 1908), but spirochaetes can actively flex themselves (Lehmann and Neumann, 1931), whereas the flexibility of other bacteria is probably only passive.

Although the organism described above lacks, at least in the living state, any obvious spirals, the following features indicate that it is a spirochaete:

1. It is sometimes possible to discern a spiral twist in non-motile organisms seen by dark ground illumination, and some specimens in smears show shallow undulations. In all other respects the morphology is typical of the tissue-inhabiting spirochaetes.

2. The organism is able actively to flex itself.

3. It is motile in the absence of demonstrable flagellae. Close examination under dark ground often suggests that it rotates, but it is difficult to be sure of this.

4. The special cultural requirements of the organism agree closely with those of the tissue-inhabiting spirochaetes, although its capacity to produce gas in artificial culture medium is not characteristic of this group.

Spirochaetes have been previously recorded from foot-rot lesions. Ludovic and Blaizot (1928) gave the name *Treponema podovis* to an organism which they described as extremely fine, as difficult to stain as *Treponema pallida*, staining pale blue with Giemsa, presenting four to five undulations in smears, and measuring 10 to 15μ in length. Blot and Lamarre (1932) also observed *T. podovis* in foot-rot lesions and described it as appearing, in dark ground, in the form of a spiral and being very motile. Howarth (1930 a and b) also observed spirochaetes in foot-rot lesions. He described them as approximately 9-11μ long by 0·1μ wide,

and presenting 3-5 undulations. The organism we have isolated differs from these descriptions in not showing undulations under dark ground illumination and usually not in smears; therefore we cannot identify it as *T. podovis*. In this laboratory a few typical *Treponemata* such as described by these workers have on rare occasions been seen in material from foot-rot lesions. Marsh and Tunnicliff (1934) searched in vain for spirochaetes such as described by these workers. They may have seen the organism described in this paper and failed to identify it as a spirochaete, thinking instead that it was *B. necrophorus*, as we did at first.

Because the organism we have isolated differs from all previously described spirochaetes in lacking obvious spirals when examined in the living state, it is regarded as a new species. It is proposed to place it in the genus *Spirochaeta*, according to the classification adopted by Hindle (1931), Lehmann and Neumann (1931), and others, and the name *Spirochaeta penortha* (paene = almost, ortha = straight) is suggested. The organism to which it bears most resemblance is *Spirochaeta phagedenis* (Noguchi, 1912).

We have hesitated before proposing a new name for an organism which may be identical with one already named, but it is thought that more confusion is likely to arise from identifying it with the descriptions quoted above, which were of organisms in mixed material.

SUMMARY.

1. An organism previously recorded by us as being constantly present in foot-rot lesions has been isolated in pure culture.

2. Its morphology, cultural characters, and pathogenicity for experimental animals are described. It grows in a medium commonly used for the cultivation of spirochaetes, but more rapidly and abundantly in culture media containing raw potato. Cultures are almost devoid of pathogenicity for experimental animals. Specific agglutination was demonstrated in high dilution with prepared anti-sera.

3. Although typically the organism lacks discernible spirals, its other characteristics necessitate its classification as a spirochaete. The name *Spirochaeta penortha* is proposed. It is possibly identical with *Treponema podovis* already described in foot-rot lesions, but it does not agree with descriptions of that organism.

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A NOTE ON THE COLORIMETRIC ESTIMATION OF COBALT IN SOLUTION BY MEANS OF NITROSO-R-SALT

by

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The colorimetric methods of estimating cobalt in solution, based upon the formation of a stable red-coloured complex with nitroso-R-salt (α-nitroso β-naphthol 3 : 6 di-sodium sulphonate), such as the method of Stare and Elvehjem (1933), are both delicate and specific.

In this method the acid test solution is treated with sodium acetate and a little nitroso-R-salt. The mixture is raised to 70° C., and concentrated potassium hydroxide solution is added until pH about 8·5 is reached. Besides cobalt, other metals (iron, nickel, copper, etc.) give coloured complexes, but the cobalt complex alone remains when the mixture is acidified with nitric acid and boiled. These other metals interfere to some extent, however, by diminishing the coloration due to cobalt and it is frequently advisable to use more nitroso-R-salt to minimize the loss.

Apart from this deficiency the method is usually considered to be satisfactory, but one of us (S.W.J.) found that it failed badly when considerable quantities of calcium chloride (several grams per milligram of cobalt) were present. Lime pellicles encased the drops of added alkali and made the titration unsatisfactory, and the losses of colour of up to 30 p.c. that were encountered may have been due, at least in part, to precipitation of cobalt hydroxide with the lime. The difficulty might be eliminated by prior removal of the calcium as sulphate from acid solution, but in view of the possible precipitation of phosphates in unknown solutions at pH 8·5, more general corrective measures have been sought.

One advantage of the method presented below is that the colour solutions are not heated at any stage of the procedure. Including those of cobalt, the hydroxides and phosphates of many of the metals that would otherwise separate are kept in solution with the aid of citrate, and to prevent the formation locally of temporary precipitates, buffered alkali is employed in the titrations.

[1] On visit from the Department of Agriculture, New Zealand.

SOLUTIONS.

Citrate mixture: 307 gm. of the salt $Na_3C_6H_5O_7,5\frac{1}{2}H_2O$ and 15 gm. of glycine are dissolved in water and diluted to 1 litre. (pH about 7).

Buffered soda: 5 gm. of NaOH and 15 gm. of glycine are dissolved in water and diluted to 100 ml. (pH about 10).

Nitroso-R-salt reagent: 1 gm. of the salt per 100 ml. aqueous solution.

Nitric acid: A 10 N solution.

Standard cobalt solution: A convenient solution contains 0·1 mg. per ml., of cobalt as chloride in 0·1 N HCl.

PROCEDURE.

The test solution should first be adjusted to pH about 1. The colour reactions are conveniently carried out in 50 ml. standard flasks at room temperature (about 20° C.). The intensity of the unknown colour solution should lie between 70 and 150 p.c. of that of the standard. Satisfactory colour standards contain several tenths of a milligram of cobalt.

(1) An aliquot of up to about 20 ml. of the test solution is mixed with 10 ml. of the citrate mixture in a 50 ml. flask. One drop of a 1 p.c. alcoholic solution of phenolphthalein is added and the mixture is titrated to a faint pink (pH about 8·5) with buffered soda, the quantity used being noted (A ml.). The volume should be adjusted to about 35 ml.

(2) 5 ml. of the nitroso-R-salt reagent are added, and the mixture is allowed to stand for 15 minutes.

(3) $7 + 0{\cdot}4A$ ml. of the nitric acid solution are added and the mixture is diluted to the mark. After 10 minutes, comparison is made with a suitable standard prepared in an analogous manner.

REMARKS.

The colour solutions are extremely stable. With the 20 ml. of citrate mixture, the presence of as much as 1·5 gm. of calcium as chloride is entirely without effect upon the colouration due to cobalt. In many instances less citrate mixture (5 ml.) and a larger aliquot of the test solution may be employed, requiring only $5 + 0{\cdot}4A$ ml. of the nitric acid.

Excess reagent always contributes a yellow component to the colour of the solutions and, as enormous excess can thereby interfere with the comparison of red intensities, it may be advantageous to use considerably less than 5 ml. when very little Co is present and interfering metals are absent. The reagent should always be present in at least 100 p.c. excess of stoichiometric requirements, which are 1 ml. to about 0·5 mg. Co, and less than 1 ml. should not be used without a compensatory increase in the time allowed for the reaction. Incidentally a 0·5 mg. Co standard with 5 ml. of reagent appears to be about 4 p.c. stronger than one with 2 ml.

When interfering metals are present it may sometimes be advantageous to use even more than 5 ml. of reagent. If their amounts have been determined it should be possible for an operator to make corrections, by conducting a series of tests with varying amounts of Co in the presence of the determined amounts of the interfering metals. The interference of 3 mg. of Ni with the coloration due to 0·5 mg. of Co when 2 ml. of reagent are used (about 6 p.c. loss), is almost eliminated when 5 ml. are used.

We are indebted to Mr. H. J. Lee, whose preparation of nitroso-R-salt was used in this work.

REFERENCE.

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THE RELATIONSHIP BETWEEN THE ACTIVITY OF TOBACCO MOSAIC VIRUS SUSPENSIONS AND HYDRION CONCENTRATION OVER THE pH RANGE 5 TO 10[1]

by

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INTRODUCTION.

In a previous publication (Best and Samuel, 1936) the relationship between pH value and the activity of suspensions of the virus of tobacco mosaic was studied over the whole pH range. The shape of the pH-activity curve on the alkaline side resembled that of a neutralization curve of an acid by a base, but the number of points on this section was not large enough to warrant any conclusions being drawn. At the time it was necessary to obtain the virus by crushing a fresh sample of leaves for each experiment and inter-comparison of results obtained in different experiments was therefore limited. Later work has shown that clarified samples of juice and solutions of purified virus may be kept for long periods (over a year) without any apparent change in concentration of active virus units. It has therefore been possible by working with a common stock suspension of virus to construct the curve covering this critical range on the basis of a larger number of points derived from a number of experiments carried out under comparable conditions. The results of these experiments are recorded in this paper.

EXPERIMENTAL METHODS.

General.

The primary lesion method of estimating relative concentrations of active virus was used throughout. The general treatment of test plants and inocula was similar to that described in previous papers. Test plants of *Nicotiana glutinosa* trimmed to three or four leaves per plant were transferred to the laboratory on the morning of the day preceding the inoculation. When surplus moisture had evaporated off the inoculated leaves the plants were transferred to the glasshouse

[1] Glasshouse facilities and part of the cost of these investigations are being provided by the Council for Scientific and Industrial Research (Australia).

for lesion development. In all cases one-half of every leaf was inoculated with a common control suspension and the other half with a test suspension of the virus.

Differences in the abrasiveness of the ground glass spatulas were eliminated in experiments where groups of plants were inoculated by changing over test and control spatulas when half the plants were inoculated. In experiments carried out on Latin square plans a similar result was obtained between the test inocula by rotating the test spatulas so that any one test system was applied on an equal number of half-leaves by each spatula. For an 8 x 8 square nine spatulas were used, one for each test system and one for the common control. The control spatula therefore received eight times the amount of wear of any test spatula, and after long usage this resulted in a falling-off in the number of lesions produced by it as compared with the test spatulas with identical inocula. Experiments performed in which the control inoculum was also included as a "test" inoculum showed that the test spatulas gave 18·3 and 15·3 per cent. more lesions than the control in two successive experiments. Consequently when this set of spatulas was used the number of lesions produced by the test inocula was reduced by 16·8 per cent. in order to bring all results to the same basis.

Source of Virus.

Values marked 1 and 2 in Table 1 were obtained by using clarified juice expressed from artificially-infected tobacco plants as source of the virus. All other values were obtained by using as source a suspension of purified virus in a buffer solution of pH 7. The virus was purified by precipitating it at its isoelectric point from the juice of leaves harvested in July, 1935.

Preparation of Inocula.

Aliquots of clarified infective juice or of a suspension of purified virus were added to the appropriate buffer solutions (0·04 M composite buffer) as previously described (Best and Samuel 1936) so that the equivalent concentration of infective juice was 1 part in 500. These suspensions were allowed to stand for 12 hours at room temperature (about 17° C.), after which an equal volume of an adjusting buffer solution was added to bring the pH values of all suspensions to that of the control (pH 7). The resulting inocula were therefore identical with respect to pH value and electrolyte content and differed only in that the virus in them had been subjected to various pH values for 12 hours. At inoculation the equivalent juice concentration was 1 in 1,000. This concentration was chosen because dilution experiments had shown that between concentrations of 1 in 1,000 and 1 in 10,000 the relationship between the relative concentration of active virus and the number of lesions was a straight line of slope 1 within the experimental error of the method. At dilutions greater than 1 in 10,000 the slope becomes less than 1. Consequently reductions in the number of lesions down to 10 per cent. of the value of the control may be taken directly as the relative concentration of active virus units, but below the 10 per cent. level the reduction is greater than is indicated by the fall in lesion number. Since the extent of this deviation is not constant no attempt has been made to apply a correction but allowance is made for it when interpreting the data.

The time of contact allowed (12 hours) was chosen in order to allow ample time for the suspensions to reach the steady state (see Best and Samuel, 1936).

All pH values were determined by means of the hydrogen electrode.

EXPERIMENTAL RESULTS.

The results of a number of experiments are brought together in Table 1, and are represented graphically in Fig. 1. The relative amounts of active virus in the suspensions after the 12-hour treatment are expressed as fractions or percentages of that in the common control of pH 7. The third figure in each determination is included for calculation purposes only and is not significant. Experience with known relative concentrations has shown that experimental values between the 100 p.c. and 20 p.c. levels are subject to an error of about ± 10 p.c. of the recorded value with the number of replicates used in this work. In order to give some idea of the density of the lesions the mean number per half-leaf is given for the controls corresponding with each test system.

TABLE 1.

The Effect of Hydrion Concentration on the Activity of Tobacco Mosaic Virus Hydrosols.

The virus was allowed to remain in contact with buffer solutions at the pH values indicated for 12 hours. At the end of this time all solutions were adjusted to pH 7 and after one hour were inoculated against the common control at pH 7.

	pH value.	$[H^+] \times 10^8$	Fraction of virus remaining active.	$\frac{[H^+]}{[\text{active virus}]} \times 10^8$	Lesions per half leaf on controls.
(1)	4·98	—	0·963	—*	12·3
(1)	5·98	—	1·171	—	10·8
(1)	6·57	—	1·059	—	12·1
(1)	7·11	—	1·013	—	11·4
(1)	7·42	—	0·986	—	14·5
(3)	7·45	—	0·986	—	20·5
(3)	7·70	—	1·038	—	22·3
(5)	7·73	1·86	0·922	2·02	23·7
(4)	7·86	1·38	0·838	1·65	12·6
(5)	7·89	1·29	0·857	1·51	26·6
(1)	7·94	1·15	0·674	1·71	14·25
(5)	8·00	1·00	0·792	1·26	18·9
(4)	8·09	0·813	0·658	1·23	15·1
(5)	8·15	0·708	0·585	1·21	23·2
(1)	8·23	0·589	0·460	1·28	11·8
(2)	8·48	0·331	0·292	1·13	28·2
(1)	8·53	0·295	0·254	1·16	13·9
(2)	8·73	0·186	0·164	1·13	28·1
(2)	8·91	0·123	0·108	1·14	28·3
(2)	9·13	0·0741	0·119	0·62	27·6
(2)	9·33	0·0468	0·066	0·71	27·6
(2)	9·56	0·0275	0·064	0·43	29·0
(2)	9·74	0·0182	0·031	0·59	29·5
(2)	10·20	0·0063	0·0055	1·15	24·4

(1) From an 8×8 Latin square on plants trimmed to 3 leaves (24 replicates per treatment).

(2) From an 8×8 Latin square on plants trimmed to 4 leaves (32 replicates per treatment).

(3) From groups of 16 four-leaved plants per treatment (64 replicates) inoculated with purified virus.

(4) and (5) As for (3) but 8 plants per treatment.

* The mean value of the constant over the pH range 8·0 to 8·9 is $1·9 \times 10^{-8}$. The mean value for the expression [active virus] $\times$ [OH′] over the same range is $0·84 \times 10^{-6}$.

It will be noticed that the fraction of virus remaining active at any pH value is lower than that recorded in a previous paper (Best and Samuel, 1936). The work there recorded was done in 1934 and it was pointed out that the estimated values of active virus may have been high. The equivalent juice concentration then used was five times that of the experiments recorded above and it is evident that in the earlier work the control inocula fell on a point on the flatter portion of the dilution curve. Qualitatively the difference is not great and for the most part negligible, but quantitatively it is of some importance.

DISCUSSION.

In general outline the pH-activity curve of Fig. 1 resembles the neutralization curve of a weak acid (dissociation residue curve). The point at which half the virus is inactivated occurs at pH 8·2. Calculation on the basis of a pK_a value of 8·2, however, does not give a good constant. If, however, the concentration of inactivated virus is ignored we obtain a good constant for the expression $\frac{[H^+]}{[\text{active virus}]}$ over the greater part of the curve and incidentally over that part of the curve where the experimental values are most reliable. This relationship, however, breaks down at the upper end of the curve, at pH values less than 8. Over this upper region the experimental curve lies about midway between the one drawn on the assumption that the above relationship holds throughout and the dissociation residue curve constructed for an acid with a pK_a value of 8·2. At the lower end of the curve (pH 9 to 10) the experimental error of estimating active virus is too high to warrant a closer analysis, but dilution curves show that the true concentration of active virus here is lower than is indicated by lesion counts. In view of the relatively large error involved a final verdict of the exact relationship must await the development of still more accurate methods of estimating the concentration of active virus. But whether either one of these is the true relationship or whether this lies between the two in the upper portions and agrees with the simpler form above pH 8, there can be no doubt that the controlling influence is the hydrion (or hydroxylion) concentration. It appears to be a fair conclusion that the inactivating effect observed is due to the neutralization of an acidic group or groups.

The inactivation is irreversible in that the virus cannot be re-activated by adjusting the pH value back to 7. It appears as if a further irreversible change follows the neutralization but it is not clear why, if this is so, the reaction does not go full forward at any pH value in the critical range. The circumstance that the "steady state" is not reached instantaneously but requires several hours for its attainment suggests the likelihood of intra-molecular changes being involved, and possibly there is a series of complex reactions in which there is finally an irreversible change to a sparingly soluble product with a constant active mass.

Another point calls for comment. The isoelectric point of this virus has been shown to fall at pH 3·4 (Best, 1935, 1936). We would therefore expect the virus to exist in an ionized condition at a pH value of 7, yet the inactivation curve

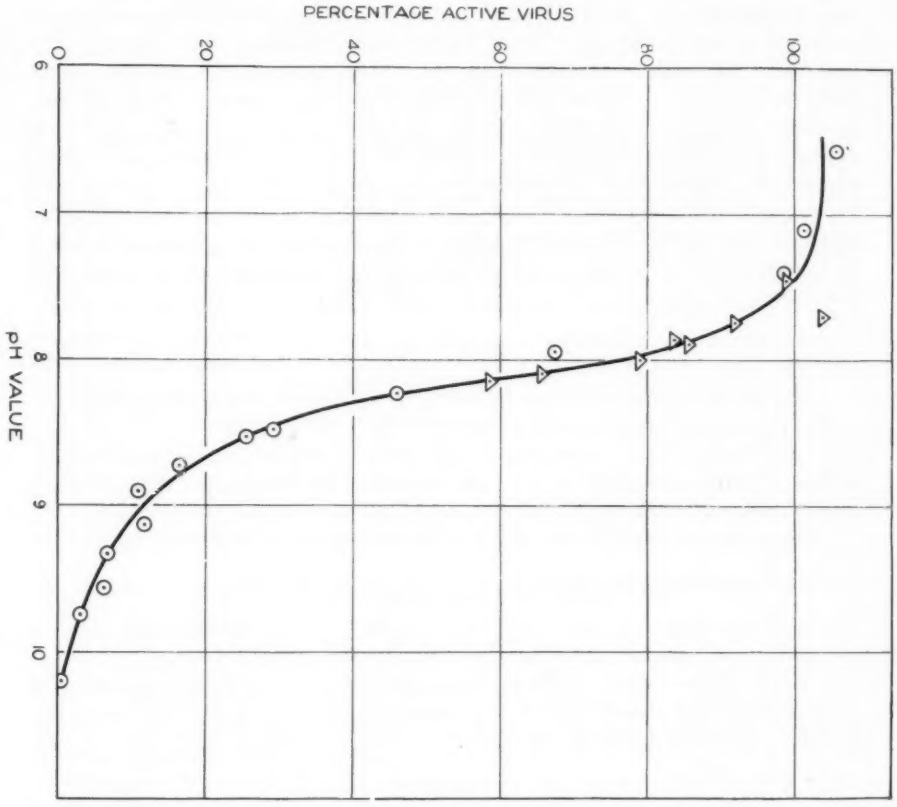

Figure 1. Showing the percentage of tobacco mosiac virus remaining active after 12 hours' storage at various pH values.

Triangles represent values obtained with purified virus.
Circles represent values obtained with clarified infective juice.
The equivalent juice concentration of all inocula was 1/1,000.

makes it fairly evident that, on the above view, the portion of the virus concerned here only begins to dissociate at a pH value above 7 and that at pH 7 this active portion of the virus molecule is unionized. It therefore seems likely that the groups entering into the reactions recorded in this paper are not concerned in the isoelectric reaction and that this latter is determined by some other portion or portions of the complex.

By analogy with current views on the nature of certain enzymes [Waldschmidt-Leitz (1936); Stern, K. G. (1936a, 1936b); Adam, N. K. (1935)] it is probable that the reactive centres of the prosthetic groups are small in comparison with the bulk of the complex and changes in other parts of this complex may lead to inactivation. It is therefore not easy to decide whether the groups inactivated are the prosthetic groups through which the virus acts, although this appears to be the most reasonable conclusion. It has been shown by Stanley (1935, 1936) and the author (1936) that this virus is protein in nature. Such different enzymes as Sumner's crystalline urease and Northrop's crystalline pepsin are protein in nature and are indistinguishable on the basis of chemical composition, and yet, as pointed out by Waldschmidt-Leitz, their specific reactive groups must be very different in nature. The virus of tobacco mosaic may best be regarded as a protein complex with a prosthetic group or groups, these latter being inactivated in weakly alkaline solutions to a definite extent, the magnitude of which is a function of the hydrion or hydroxylion concentration.

SUMMARY.

The percentage inactivation of the virus of tobacco mosaic has been determined for various pH values over the pH range 5 to 10.

Inactivation of the virus set in at a pH value of about 7·8 and the fraction inactivated was progressively larger with increasing pH values until at a pH value of 10·2 only about one-half of a per cent. of the virus added remained active.

Between pH values of 8·0 and 8·9, corresponding with 21 p.c. and 90 p.c. inactivation respectively, the ratio $\frac{[H^+]}{[\text{active virus}]}$ was found to be a constant but this relationship did not hold at pH values below 8. It is concluded that inactivation of the virus is associated with the neutralization of acidic groups.

The bearing of these results on the nature of the virus is discussed and it is suggested that the groups thus neutralized are an integral part of the chemically reactive prosthetic groups of the virus.

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PHOSPHATASES AND HEXOSEPHOSPHATE IN THE BANANA

by

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In 1933 Nelson and Hemperley attempted to show the presence of an enzyme capable of causing the hydrolysis of phosphoric esters in the banana. They added a solution of hexose diphosphoric ester to banana pulp or extract which, after standing for some hours, showed an increase in free phosphate. This work is open to the criticism that combined phosphate exists in the banana and that the increase in free phosphate observed might be accounted for by hydrolysis of this compound.

On this account, sodium glycerophosphate and sodium β-glycerophosphate have been added to the banana pulp used in the experiments described in this communication, and a suitable technique has been elaborated to detect hydrolysis of these compounds. Further, an attempt has been made to isolate and identify the organic phosphorus compounds normally present in the banana.

EXPERIMENTS ON PHOSPHATASES.

(a) *Material Employed.*

In the experiments recorded below, bananas of the variety *Musa Cavendishii* were used. The fruits were ripened by normal commercial methods and were withdrawn from the ripening chambers and used for experimental purposes at the "spring" stage, which coincides with the period of most active metabolism of the fruit. At this stage, the pulp is beginning to soften but no yellow colour has appeared in the skin.

The skins were removed from the bananas and the pulp was macerated to a homogenous mass in a mortar.

(b) *The Presence of Phosphatase.*

In one experiment, to two lots (a) and (b) of 23 gm. of banana pulp were added 2 ml. of 4 p.c. solution of sodium glycerophosphate (B.D.H.), containing no free phosphate, together with 100 ml. of water and a few drops of toluene to inhibit microbial growth. To lot (a), 25 ml. of 6 p.c. solution of trichloracetic acid were added immediately to precipitate proteins and to inhibit enzyme action, while lot (b) was allowed to stand for a period of 23 hours before the trichloracetic acid was added. The mixtures were then made up to 200 ml. with water and filtered, and the free phosphate was estimated colorimetrically in each by the method of Martland and Robison (1926). The amounts of free phosphate as phosphorus per ml. in lots (a) and (b) were 0·018 mg. and 0·066 mg., respectively. The nett increase was, therefore, 0·048 mg.

In another experiment, the pulp of the bananas was minced and mixed, ten lots each of 23 gm. being used. To lot 1, 25 ml. of 6 p.c. trichloracetic acid were added, together with 2 ml. of a solution of sodium glycerophosphate containing 3·695 mg. of phosphorus per ml. Water was added to give a volume of 250 ml., and the free phosphate was estimated in the filtrate as before. To each of the other nine lots were added 2 ml. of the glycerophosphate solution, 100 ml. of water, and a few drops of toluene. The mixtures were held at a temperature of 20°C., and 25 ml. of the trichloracetic acid solution were added successively to the mixtures at gradually increasing intervals of time. After this addition, the mixtures were made up to 250 ml., and the free phosphate was estimated. The results are shown in table 1 and graphically in fig. 1.

TABLE 1.

Lot. No.	Time of Action (Hours).	Free phosphate as mg. of phosphorus per ml.
1	0	0·016
2	½	0·017
3	1½	0·022
4	2½	0·027
5	4	0·031
6	5½	0·034
7	7½	0·039
8	24	0·049
9	31½	0·050
10	48	0·050

The data presented in Table 1 demonstrate the progressive production of free phosphate, and reference to Fig. 1 shows that the curve obtained by plotting free phosphate against time of reaction has a form typical of enzyme action.

The following analysis of the above results shows that the free phosphate has been derived, in part, from the added combined phosphate. 23 gm. of the banana pulp used contained:

Total phosphate as phosphorus	6·9 mg.
Free „ „ „	3·9 mg.
Combined „ „ „	3·0 mg.

To each portion of pulp 7·4 mg. of phosphorus as glycerophosphate were added, making the total combined phosphorus 10·4 mg. After an incubation period of 48 hours an increase of free phosphate equivalent to 8·5 mg. of phosphorus had occurred, representing 81 p.c. of the total combined phosphate of the mixture. It is obvious, therefore, that of the free phosphate, at least 5·5 mg. (as phosphorus) must have been derived from the added glycerophosphate.

In a further experiment, three portions each of 23 gm. of banana pulp were treated as follows:

(a) heated to 100° C.;
(b) 25 ml. trichloracetic acid added;
(c) untreated control.

Two ml. of glycerophosphate and 100 ml. of water were then added to each portion, and, after 24 hours, 25 ml. trichloracetic acid were added to portions (a) and (c), the volumes of each being made up to 250 ml.

The concentrations of free phosphate as phosphorus per ml. were determined as above, being respectively 0·014 mg., 0·013 mg., and 0·056 mg. The hydrolytic activity of the banana pulp was thus destroyed by boiling, providing good evidence that it is due to enzyme action.

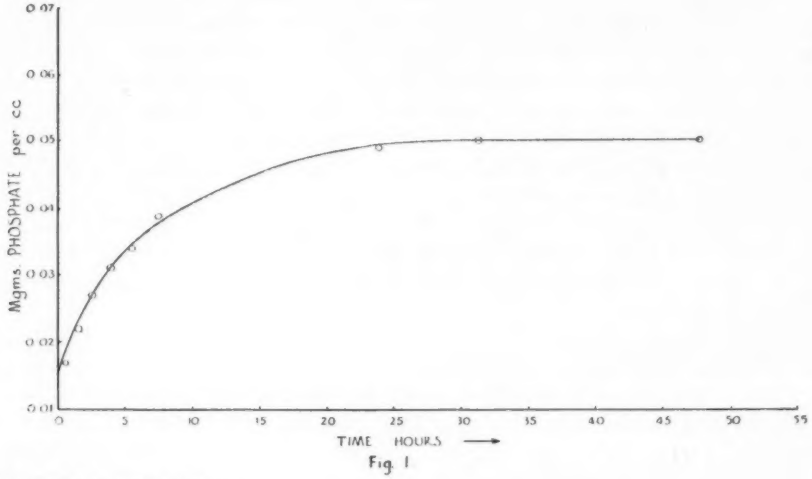

Fig. 1

(c) *Tests for β-phosphatase.*

Two mixtures were made, each containing 23 gm. banana pulp, 2 ml. of 4 p.c. solution of pure sodium β-glycerophosphate, 100 ml. water, and a few drops of toluene. Twenty-five millilitres of 6 p.c. trichloracetic acid were added immediately to portion (a) and to portion (b) after it had been stored at 200°C. for 20 hours. Both mixtures were diluted to 250 ml. with water, filtered and the free phosphate (as phosphorus per ml.) estimated in the filtrate as before. The concentrations found were 0·022 mg. and 0·060 mg. respectively, thus demonstrating the presence in banana pulp of an enzyme capable of hydrolysing β-glycerophosphate.

In a further experiment, using additional controls, the mixtures employed were as follows:

(a) 23 gm. banana pulp, 2 ml. of 4 p.c. solution of sodium β-glycerophosphate, 100 ml. water, a few drops of toluene, and 25 ml. of 6 p.c. trichloracetic acid added immediately.
(b) As for (a), but the trichloracetic acid added after 22 hours at 20°C.
(c) As for (a), but containing no β-glycerophosphate.
(d) As for (b), but containing no β-glycerophosphate.

The figures for total free phosphate (as mg. of phosphorus) in these mixtures were 3·5, 11·5, 3·5, and 4·52 respectively.

Since the amounts of total phosphate (as phosphorus) in 23 gm. of banana pulp and in 2 ml. of 4 p.c. sodium β-glycerophosphate were 5·9 mg. and 7·61 mg. respectively, the total combined phosphate (as phosphorus) at the commencement of the experiment was therefore [(5·9—3·5+7·61] mg. or 10·01 mg., and the increase in free phosphate (as phosphorus) equalled (11·5—3·5) mg. or 8·0 mg.

This increase represents 80 p.c. of the total combined phosphate, and at least 5·6 mg. must have been derived from the added glycerophosphate.

Isolation of Hexose Phosphates from the Banana.

The pulp from 400 bananas at the "spring" stage was minced, an equal quantity of sand added and the whole ground in a mortar. To the juice expressed by means of a hand press, 600 ml. of 10 p.c. trichloracetic acid solution were added, and the precipitate filtered off through glass wool. To this protein-free filtrate was added 0·5 gm. of barium acetate and the reaction of the liquid was then adjusted to pH 8·6 by the addition of baryta, the bulk of which was added in hot, saturated solution, the final adjustment being made with cold saturated solution. The precipitate was removed by filtration, washed first with a little water, then absolute alcohol and dried *in vacuo*. This precipitate should contain the bulk of any hexose diphosphate present together with barium phosphate resulting from the presence of any inorganic phosphate, while hexose monophosphate should be present in the filtrate.

Employing the method of Robison and Morgan (1930), the above precipitate was extracted with 200 parts of water adjusted with baryta to pH 8·6, at a temperature of 0° C., and the insoluble matter was filtered off. A test at this stage indicated the absence of inorganic phosphate in the filtrate. To this filtrate alcohol was added until a slight cloud formed. Portions, 200 ml. in volume, were heated to 70° C. in a water bath and filtered hot. The resulting precipitate was dried at a low temperature and weighed 1·0 gm. A further, less pure, precipitate weighing 0·2 gm. was obtained by adding more alcohol to the filtrate and re-heating to 70° C. Some of the properties of these precipitates are as follows:

(i) The precipitate was heated with concentrated nitric acid, cooled, and ammonium molybdate added. The mixture was then warmed to 65°C. when a yellow precipitate formed indicating the presence of phosphate, which must have been present in the original precipitate as organic phosphate since no test for phosphate was successful until after hydrolysis had been effected.

(ii) On treating some of the precipitate with a solution of sodium sulphate and filtering, the filtrate reduced Fehling's solution on boiling, and also after standing in the cold.

(iii) Seliwanoff's test was positive, indicating the presence of a fructose derivative.

(iv) An osazone crystallizing in yellow needles was prepared by adding to a solution of the sodium salt of the supposed hexose phosphate prepared from the barium salt, a solution of phenyl hydrazine base in acetic acid and heating for half an hour in a water-bath. Insufficient osazone was obtained to carry out an analysis but, after prolonged heating with sulphuric and nitric acids, it gave a positive test for phosphate.

There was insufficient material for a satisfactory measurement of the optical rotation.

There is some evidence therefore, to indicate the presence of a fructose diphosphate in the banana pulp.

In order to isolate only hexose monophosphate, a slight excess of basic lead

acetate was added to the liquid obtained after the removal of mixed barium phosphate and hexose diphosphate. The resulting precipitate was washed with water and decomposed by hydrogen sulphide; the lead sulphide was filtered off, and the filtrate was freed from hydrogen sulphide by aeration. The latter filtrate was strongly acidic in reaction and did not contain inorganic phosphate. A few drops of 25 p.c. sulphuric acid were added to a portion of this filtrate and the whole heated. Examination of the solution showed that it possessed definite dextrorotation and that it contained inorganic phosphate. The reaction of the remainder of the filtrate was, therefore, adjusted by baryta to pH 8·4 and poured into two volumes of absolute alcohol; the precipitated barium salts were washed with alcohol and rapidly dried *in vacuo*. The precipitate had the following properties:

(i) The filtrate obtained after treatment with sodium sulphate solution reduced Fehling's solution.

(ii) It contained phosphoric acid which could be set free by hydrolysis with acid.

(iii) The filtrate from treatment with sodium sulphate, when heated with phenyl hydrazine acetate, gave a crystalline osazone which contained phosphoric acid.

Too little material was obtained for complete analysis, but the evidence points to the presence of a hexosemonophosphate yielding a dextro-rotatory sugar on hydrolysis.

SUMMARY.

1. The pulp of Cavendish banana contains an enzyme hydrolysing glycerophosphoric acid.

2. It also contains a β-phosphatase.

3. There is some evidence that during the ripening process both fructose diphosphate and a hexosemonophosphate are present.

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